IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of STEINMAN *et al.*

Atty. Docket No. ARG010RC	Confirmation No. 9977
Application No. 09/073,596	Art Unit: 1644
Date Filed: 6 May 1998	Examiner: Gerald R. Ewoldt

Title: METHOD FOR IN VITRO PROLIFERATION OF DENDRITIC CELL PRECURSORS AND THEIR USE TO PRODUCE IMMUNOGENS

* * *

BRIEF FOR APPEAL UNDER 37 CFR § 41.37

July 20, 2010

Mail Stop Appeal Brief—Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Appellants submit this Brief to appeal the Examiner's final rejections as set forth in the Office Action mailed February 23, 2010 (the "final Office Action"). The fee required under 37 CFR § 41.20(b)(2) is submitted herewith. This application is now entitled to small entity status.

The Notice of Appeal was filed on May 20, 2010. This Brief is timely filed.

Reversal of the Examiner's rejections of claims 99, 101, 103-113, 116, 120 and 142-145 by the Board of Patent Appeals and Interferences (the "Board") is respectfully requested.

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I.   REAL PARTY IN INTEREST

Argos Therapeutics, Inc., and The Rockefeller University are the assignees of rights in the subject application, as well as the invention disclosed and claimed therein, by virtue of the assignments recorded in the PTO on: September 21, 2001, starting at reel 012199 and frame 0923 (assignment from inventor Gerold Schuler to Merix Bioscience, Inc.; also recorded on September 10, 2003 at reel 013961 and frame 0748); April 30, 2008, starting at reel 020876 and frame 0811 (assignment from inventors Ralph Steinman and Kayo Inaba to The Rockefeller University); and documentation of change of name of Merix Bioscience, Inc. to Argos Therapeutics, Inc. recorded in the PTO on February 15, 2007 at reel 018942 and frame 0265. Geron Corp. holds a license to certain rights in the application. Argos Therapeutics, Inc. has power of attorney for this application as recorded on November 18, 2004.

II.   RELATED APPEALS AND INTERFERENCES

Appellants and their legal representative do not know of any prior or pending appeal, interference, or judicial proceeding which is related, directly affects, or is affected by the Board's decision in this appeal. Notices of Appeal were filed for this application on July 9, 2001 and August 14, 2003, but the appeal process was not continued; instead, prosecution was re-entered.

III. STATUS OF CLAIMS

Claims 99, 101, 103-113, 116, 120 and 142-145 stand rejected. They are at issue in this appeal and listed in the Claims Appendix.

Claims 1-98, 100, 102, 103, 114, 115, 117-119, and 121-141 were canceled without prejudice or disclaimer.

IV. STATUS OF AMENDMENTS

No amendment was filed subsequent to final rejection.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The invention involved in this appeal is directed to an *in vitro* composition comprising mature dendritic cells expressing modified antigen and derived from an *in vitro* culture of an enriched and expanded population of proliferating dendritic cell precursors by a specified method that comprises, *inter alia*, a step of culturing the tissue source on a substrate in a culture medium comprising GM-CSF; see, *e.g.,* page 25, lines 19-23, which explain that:

> "GM-CSF has surprisingly been found to promote the proliferation in vitro of precursor dendritic cells. Cells are cultured in the presence of GM-CSF at a concentration sufficient to promote the survival and proliferation of dendritic cell precursors."

Support for independent claims 101, 120, and 145 is found, *inter alia*, in the first paragraph of the Detailed Description (page 19, lines 25-31), which explain that:

> "This invention relates to a method of producing cultures of proliferating dendritic cell precursors which mature *in vitro* to mature dendritic cells. The dendritic cells and the dendritic cell precursors produced according to the method of the invention may be produced in amounts suitable for various immunological interventions for the prevention and treatment of disease."

The specification explains in the "Summary of Invention" (page 9, line 35 through page 10, line 3) that:

> "Another embodiment of the invention [is] antigen-activated dendritic cells prepared according to the method of the invention in which antigen-activated dendritic cells have been exposed to antigen and express modified antigens for presentation to and activation of T cells."

The specification also describes (*e.g.*, on page 40, lines 25-28) the benefits of the invention:

> "The present invention provides for the first time a method of obtaining dendritic cells in sufficient quantities to be used to treat or immunize animals or humans with dendritic cells which have been activated with antigens".

As can be seen, for example, from these passages, slightly different terminology is used in some parts of the specification to describe these dendritic cells that have been activated with antigens.

Support for the method steps set forth in claims 101, 120, and 145 can be found, *inter alia*, in the specification on page 8, line 30 through page 9, line 10 and on page 9, line 35 through page 10, line 4, which are as follows, respectively:

> "This invention also provides a method of producing *in vitro* mature dendritic cells from proliferating cell cultures. The method comprises (a) providing a tissue source comprising dendritic cell precursor cells; (b) treating the tissue source from (a) to increase the proportion of dendritic cell precursors in order to obtain a population of cells suitable for culture in vitro; (c) culturing the tissue source on a substrate in a culture medium comprising GM-CSF, or a biologically active derivative of GM-CSF, to obtain non-adherent cells and cell clusters; (d) subculturing the nonadherent cells and cell clusters to produce cell aggregates comprising proliferating dendritic cell precursors; (e) serially subculturing the cell aggregates one or more times to enrich the proportion of dendritic cell precursors; and (f) continuing to culture the dendritic cell precursors for a period of time sufficient to allow them to mature into mature dendritic cells."

> "Another embodiment of the invention are antigen-activated dendritic cells prepared according to the method of the invention in which antigen-activated dendritic cells have been exposed to antigen and express modified antigens for presentation to and activation of T cells."

Additional support for the step of exposing the cells to antigen can be found, for example, on page 34, line 34 through page 35, line 9 and page 36, line 31 through page 37, line 4.

Dependent claim 104 (specifying that the tissue source is blood) is supported by page 23, lines 17-19; dependent claim 107 (specifying that the concentration of GM-CSF in the culture medium is about 30-100 U/ml) by page 25, lines 29-13; dependent claim 112 (specifying that the

tissue source is treated to remove blood cells) by page 20, lines 34-36 and page 21, lines 10-12; dependent claim 105 (specifying that the tissue source is bone marrow) by page 21, lines 19-21; dependent claim 108 (specifying that the concentration of GM-CSF in the culture medium is about 500-1000 U/ml) by page 26, lines 1-6; dependent claim 113 (specifying that the tissue source is treated to remove B cells and granulocytes) by page 21, line 19 through page 22, line 3; dependent claim 106 (specifying that GM-CSF is present in the culture medium at a concentration of about 1-1000 U/ml) by page 25, lines 27-29; dependent claim 109 (specifying that the cell aggregates are blood-derived and are subcultured from about one to five times) by page 29, lines 21-35; dependent claim 110 (specifying that the cell aggregates are subcultured one to five times) by page 29, lines 34-36; dependent claim 111 (specifying that the culture medium is selected from a specified group and is supplemented with serum) by page 25, lines 2-6; dependent claim 116 (drawn to a pharmaceutical composition comprising the composition of claim 101) by page 42, lines 10-23; dependent claim 99 (specifying that the dendritic cells express an amount of the modified antigen to provide between about 1 to 100 micrograms of the modified antigen) by page 42, lines 23-25; dependent claim 142 (specifying that the dendritic cell precursors are human) by, *e.g.*, page 12, lines 18-22 and page 28, lines 3-6; dependent claim 143 (specifying that the dendritic cell precursors are obtained from blood) by page 23, lines 17-19; and dependent claim 144 (specifying that the dendritic cells are obtained from bone marrow) by page 21, lines 19-21. Additional support for these claims can generally also be found in the specification.

Therefore, the invention as presently claimed is clearly supported by the disclosure as originally filed.

VI.   GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

A.   Was it proper to deny the benefit of the priority claim to the application? (no statutory basis for this decision was specified in the Office Action, although it seems like a rejection under 35 U.S.C. § 112, first paragraph)

B.   Under 35 U.S.C. § 102(a), was it proper to reject claims 99, 101, 104-113, 116, 120, and 142-145 as allegedly anticipated?

C.   Under 35 U.S.C. § 103(a), was it proper to reject claims 99, 101, 104-113, 116, 120, and 142-145 as allegedly obvious?

D.   Under 35 U.S.C. § 112, first paragraph, was it proper to reject claims 99, 101, 104-113, 116, 120, and 142-145 as allegedly containing new matter?

E.   Under 35 U.S.C. § 112, second paragraph, was it proper to reject claim 120 as allegedly indefinite?

VII.   ARGUMENTS

The claims do not all stand or fall together because the Board may reverse in part the denial of the benefit of the priority claim and the rejection under 35 U.S.C. §112, first paragraph, and may come to different conclusions for independent claims 101, 120, and 145. Claims 99, 104-113, 116, and 142-144 depend from or incorporate the limitations of independent claim 101 and stand or fall together with independent claim 101. The rejections of independent claim 120 and independent claim 145 may be reversed or affirmed separately from each other and separately from claim 101 and its dependent claims.

Claim 120 is the only claim rejected under 35 U.S.C. §112, second paragraph, and so this rejection may be affirmed by the Board even if the other rejections are reversed.

Priority Claim

The disclosure as originally filed need not provide "*in haec verba* support for the claimed subject matter at issue," rather, the disclosure should convey to one skilled in the art that the inventor had possession of the invention at the time of filing. *Purdue Pharma L.P. v. Faulding, Inc.*, 230 F.3d 1320, 1323 (Fed. Cir. 2000). The Patent Office has the initial burden of presenting evidence or a reason why persons of ordinary skill in the art would not have recognized such a description of the claimed invention in the original disclosure. See *In re Gosteli*, 872 F.2d 1008, 1012 (Fed. Cir. 1989).

A. The final Office Action states that the priority claim for the present application is denied because, allegedly, "the applications do not disclose the invention of the instant claims." No claim numbers or statutory basis for the denial of the priority claim are specified, so these remain unclear. However, the statements in the Office Action regarding the denial of the priority claim appear to be based on the written description requirement of 35 U.S.C. § 112, first paragraph, and appear to apply to all of the claims, they will be addressed as if this had been explicitly stated in the Office Action.

Support in the present specification for each claim is discussed in the Summary of Claimed Subject Matter (section V) above. As can be seen, for example, from these passages, slightly different terminology is used in some parts of the specification to describe these dendritic cells that have been activated with antigens. Nevertheless, the specification when read as a whole fully supports all of the present claims. Corresponding support is found in the priority application (the "'612 application), as detailed for all of the claims in the chart provided in Appendix A; a copy of the '612 application is provided as Appendix B. The language of the pending claims has been drafted to conform as closely as possible to the exact language used in the present specification and to the language of the '612 priority application, both of which fully support the present claims. The support for the claims in the '612 application cited in Appendix A is also found in every application in the priority chain, including the instant application (No. 09/073,596).

The claimed invention is described throughout the present application and relates to compositions and methods for providing them which involve culturing dendritic cell precursors *in vitro* in the presence of GM-CSF, which ultimately produces mature dendritic cells. The invention also provides methods and compositions that further involve exposing the cells to antigen, which produces antigen-activated mature dendritic cells that express modified antigens. The mature dendritic cells and the antigen-activated mature dendritic cells as well as the methods of producing them are all embodiments of the invention. The specification discusses these various aspects of the invention throughout, particularly, for example, in the Summary of the Invention (page 8, line 13 through page 10, line 28); further embodiments and aspects of the invention are described in the remainder of the same Summary of the Invention section.

Antigen-activated dendritic cells (also referred to, *inter alia*, as dendritic cells expressing modified antigen) are dendritic cells that are prepared according to the methods of the invention and are further (*i.e.*, *additionally*) treated by exposure to antigen. That is, "[a]ntigen-activated dendritic cells [can be] prepared according to the method of the invention in which antigen-activated dendritic cells have been exposed to antigen and express modified antigens for presentation to and activation of T cells" (specification at page 9, line 35 through page 10, line 3). In other words, the methods of the invention are equally applicable to "antigen-activated dendritic cells," but additional steps beyond the standard methods are necessary to produce these activated cells.

A reading of the specification as a whole reveals that the specification describes various embodiments of the invention and fully supports the present claims. Corresponding support is also found in the priority application. Accordingly, the benefit of the priority claim should be accorded to the present application and the Board should reverse the denial of the priority claim.

35 U.S.C. §102—Anticipation

A claim is anticipated only if each and every limitation as set forth in the claim is found, either expressly or inherently described, in a single prior art reference. *Verdegaal Bros. v. Union Oil Co. of Calif.*, 814 F.2d 628, 631 (Fed. Cir. 1987).

B. The final Office Action alleges that claims 99, 101, 104-113, 116, 120, and 142-145 are anticipated under 35 U.S.C. § 102(a) over Pancholi *et al.* (1992) *Immunology* 76: 217-224 ("Pancholi"). As noted in the Office Action, the Pancholi reference is not available as prior art if the priority claim for the present application is accorded full weight because the Pancholi reference was published in June 1992, after the filing date of the '612 priority application, which was April 1, 1992. As discussed above under part A of "Arguments," the claimed subject matter is fully supported by the priority application. Accordingly, the Pancholi reference is not available as prior art and this rejection should be reversed by the Board.

Further, even if Pancholi were available as a reference, it would not anticipate the cells of the present claims. All of the claims specify dendritic cells derived from an *in vitro* culture of dendritic cell precursors. As described in the specification, the cells of the invention are cultured in GM-CSF, which was surprisingly found to promote the proliferation *in vitro* of precursor dendritic cells; these precursors can be used to provide large populations of antigen-activated dendritic cells. Pancholi merely teaches the isolation of dendritic cells directly from blood. Thus, the process used to make the claimed cells is different from the process described by Pancholi, and the cells that result from each process are also different in at least two significant ways. First, the present invention provides dendritic cells derived from an *in vitro* culture of a population of enriched and expanded proliferating precursor cells, thereby overcoming the previously existing problem in the art of not being able to obtain sufficient quantities of dendritic cells for clinical treatment (as discussed in the present specification, *e.g.*, on page 2, lines 21-26 and page 8, lines 3-11). Second, dendritic cells prepared according to the claimed methods are more effective at presenting antigen to T cells *in vitro* than the cells reported by Pancholi.

Particularly, results obtained with dendritic cells prepared according to the claimed methods show significant stimulation of T cells at a dendritic cell to T cell ("DC:T") ratio of 1:1000. This is shown, for example, in Figure 15A, which presents the results of an experiment in which T cells respond to stimulation by immature or mature BCG-pulsed dendritic cells prepared according to the methods of the invention at DC:T cell ratios of 1:100 and 1:1000. In contrast, Pancholi's DCs show no stimulation of T cell response at a much lower DC:T ratio of 1:100 (see Figure 2b). Thus, the cells of the present invention are more effective at presenting antigen to T cells *in vitro* than the cells taught by Pancholi and are therefore different from the cells taught by Pancholi. Because the cells taught by Pancholi are different from the claimed cells, the Pancholi reference would not anticipate the claims even if it were available as prior art, which it is not. Accordingly, this rejection should be reversed by the Board.

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35 U.S.C. §103—Obviousness

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"[T]he Examiner bears the burden of establishing a prima facie case of obviousness based upon the prior art." *In re Fritch*, 972 F.2d 1260, 1265 (Fed. Cir. 1992). "[A] patent composed of several elements is not proved obvious merely by demonstrating that each of its elements was, independently, known in the prior art." *KSR Int'l. Co. v. Teleflex, Inc.*, 550 U.S. 398, 418 (2007). "[I]t can be important to identify a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention does." *Id.* "Rejections on obviousness grounds cannot be sustained by mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness." *Id.* at 417-418 (quoting *In re Kahn*, 441 F.3d 977, 988 (Fed. Cir. 2006)).

In determining the differences between the prior art and the claims, the question under 35 U.S.C. § 103 is not whether the differences themselves would have been obvious, but whether the invention as a whole would have been obvious. See, *Stratoflex, Inc. v. Aeroquip Corp.*, 713 F.2d 1530 (Fed. Cir. 1983); *Schenck v. Nortron Corp.*, 713 F.2d 782 (Fed. Cir. 1983). When assessing the patentability of product-by-process claims over the prior art, the structure implied by the process steps should be considered, especially where the product can only be defined by the process steps by which the product is made, or where the manufacturing process steps would

be expected to impart distinctive structural characteristics to the final product. See *In re Garnero*, 412 F.2d 276, 279 (CCPA 1979).

C. The final Office Action alleges that claims 99, 101, 104-113, 116, 120, and 142-145 are obvious under 35 U.S.C. § 103(a) over Inaba *et al.* ((1990) *J. Exp. Med.* 172: 631-640) in view of Steinman *et al.* ((1988) *Ann. N.Y. Acad. Sci.* 546: 80-90) and Markowicz and Engleman ((1990) *J. Clin. Invest.* 85: 955-961).

The Office Action concludes (page 5, last paragraph of block quote) that

> "It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to add GM-CSF to a cell culture of DCs [(dendritic cells)]such as the mouse cultures of Inaba et al. and Steinman et al. or the human cultures of Steinman et al. and Markowicz and Engleman. The ordinarily skilled artisan would have added GM-CSF to DC cultures given the teachings of Steinman et al., that, DC 'maturation is driven by factors such as IL-1 and GM-CSF,' etc. and Markowicz and Engleman, that, 'GM-CSF ... profoundly affects the morphology and viability of DCs isolated from peripheral blood....' Accordingly, the GM-CSF-cultured DCs as claimed are obvious in view of the combined prior art."

Appellants respectfully disagree with this rejection because none of the references, nor any combination thereof, teaches the claimed compositions or the method steps specified in the claims that are necessary to produce them. The Inaba reference includes experiments on dendritic cells but does not teach anything about GM-CSF and also does not teach or suggest that dendritic cell precursors even exist. None of the references, either alone or in any combination, teach or suggest that the use of GM-CSF to culture dendritic cell precursors *in vitro* produces an enriched and expanded population of proliferating dendritic cell precursors that can be used to produce a large population of mature dendritic cells expressing modified antigen.

The Office Action cites the Markowicz and Engleman reference as teaching that "'GM-CSF ... profoundly affects the **morphology and viability** of DCs isolated from peripheral blood...'" and concludes that one of skill would have been motivated to add GM-CSF to DC cultures. However, rather than suggesting such a combination, the Markowicz and Engleman reference instead teaches away from the claimed invention and particularly teaches away from the use of GM-CSF to induce proliferation. Particularly, Markowicz and Engleman conclude that in the presence of GM-CSF, "the number of viable cells as well as the number of branched cells per well **remained stable over time, suggesting that GM-CSF does not cause DC to**

divide and proliferate" (sentence bridging page 958-959; emphasis added). This is supported by Markowicz and Engleman's Figure 4 (on page 959), which shows that there was no significant increase in the number of viable cells (as well as differentiated DC) "throughout the culture period" (see Figure 4 legend on page 959). In view of this teaching away by Markowicz and Engleman, one of skill in the art would not have added GM-CSF to induce cell proliferation of Inaba's cell cultures and so would not have been motivated to produce the claimed invention.

The third reference cited in this rejection of claims is Steinman *et al.* ((1988) *Ann. N.Y. Acad. Sci.* 546: 80-90), which is cited in the Office Action as teaching:

> "the enrichment and culturing of both mouse and human immature DCs found in blood, as well as bone marrow (see pages 81-83) and that, 'maturation is driven by factors such as IL-1 and GM-CSF' (see page 83). The reference further teaches that 'GM-CSF is critical in mobilizing active DCs at the onset of a cell-mediated immune response' (see page 88)."

Appellants disagree and respectfully submit that the Steinman reference teaches away from the claimed invention because it states that GM-CSF has a role *in vivo* in **maturation** of cells in certain tissues and proposes that GM-CSF may be involved in **mobilizing** DCs at the onset of a cell-mediated immune response. The Steinman reference does not teach or suggest that culture of dendritic cell precursors in GM-CSF can produce a population of proliferating dendritic cell precursors as taught by Applicants, or the mature dendritic cells resulting from such a process, as presently claimed.

The Steinman reference also discusses "immature forms of dendritic cells," but states that (paragraph bridging pages 83 and 84):

> "The term 'immature' is used, because these populations must be cultured for 1-2 days before optimal levels of surface Ia and accessory function are expressed."

That is, the Steinman reference teaches that "immature" DCs need only be cultured for 1-2 days, and that this time is necessary for maturation. Thus, while discussing "immature" DCs and, separately, a role for GM-CSF in certain DC functions, the Steinman reference does not teach or suggest that GM-CSF plays any role in stimulating cell proliferation of DCs, particularly of dendritic cell precursors, and thus does not teach or suggest the claimed invention.

When assessing the patentability of product-by-process claims over the prior art, the structure implied by the process steps should be considered, especially where the product can

only be defined by the process steps by which the product is made, or where the manufacturing process steps would be expected to impart distinctive structural characteristics to the final product. See *In re Garnero*, 412 F.2d 276, 279 (CCPA 1979). Here, the claims are drawn to an *in vitro* composition of cells derived from an *in vitro* culture of an enriched and expanded population of proliferating precursors by a method comprising culture in GM-CSF, which surprisingly was found to promote that proliferation; further, these cells are cultured *in vitro* in the presence of antigen and express modified antigen.

The claimed cells differ from previously reported cells, for example, in their ability to take up antigen even after extended periods of culture. Specifically, for example, the fresh spleen cells taught by the cited Inaba reference differ in at least several significant ways from the *in vitro* compositions of the present invention. First, as taught by the Inaba reference ("Inaba"), fresh spleen cells can only take up antigens for a short time, and lose this ability in culture. The Inaba reference states:

> "As will be evident in Results, it was necessary to expose fresh rather than cultured dendritic cells to a foreign protein to successfully charge these APC with antigen."

(see Inaba *et al.* (1990), *e.g.*, at page 632, left column, first full paragraph)

Further, Inaba *et al.* state:

> "We conclude that freshly isolated dendritic cells can be successfully pulsed with a variety of soluble protein antigens *in vitro*, but that it is important to administer the antigen shortly after isolating the dendritic cells from the spleen."

(see Inaba *et al.* (1990) at page 632, right column, first paragraph of "Results" section).

In contrast to the cells taught by Inaba, the cells of the present invention can take up antigen after being cultured for many days (see, *e.g.*, Figure 13, showing uptake and expression of antigen after cells had been cultured for 6 days in GM-CSF). Because fresh spleen cells like those of Inaba's lose their ability to take up antigen in culture, they cannot give rise to enriched and expanded cell populations which take up and then express modified antigen to which they have been exposed *in vitro*, as required by the present claims. The cells of the present invention provide enriched and expanded cell populations in clinically useful quantities, a benefit resulting from Appellants' innovative step of culturing the cells in GM-CSF so as to obtain proliferating dendritic cell precursors. Thus, the claimed compositions provide a number of advantages that

result from the novel methods of their production, which are not taught or suggested by the prior art.

As further evidence of nonobviousness, Appellants note that the Examiner previously withdrew an obviousness rejection over the cited Inaba reference for the stated reason that "an objective and quantifiable difference between the DCs of the prior art and the DCs of the instant claims was established (the inability of the DCs of Inaba et al. to capture antigen after several days of culture)" (see the Final Office Action of November 18, 2008, page 5). This obviousness rejection was later withdrawn (in the Office Action of 13 August 2004) because Appellants' "arguments that the cells of the instant invention are not the cells of the reference because the cells of the reference were not cultured in GM-CSF, has been found convincing." Now, however, the present obviousness rejection similarly cites references (including the same Inaba reference) that do not teach or suggest the use of GM-CSF to culture dendritic cell precursors *in vitro* to produce an enriched and expanded population of proliferating dendritic cell precursors, nor that these cells can be used to produce a large population of mature dendritic cells expressing modified antigen. In view of this as well as the discussion above, Appellants respectfully submit that the Examiner has not made out a *prima facie* case of obviousness.

In summary, culture of the dendritic cell precursors in GM-CSF, as discovered by Appellants, is essential to the development of *in vitro* cultures of proliferating precursor cells and provides a number of advantageous properties to the resulting dendritic cells, including embodiments where the cells have been cultured *in vitro* in the presence of an antigen and give rise to mature dendritic cells expressing modified antigen as required by the claims. None of the cited references teaches or suggests this critical feature of the methods that produce the claimed cells. Accordingly, the claimed invention is not rendered obvious by any of the cited references or any combination thereof and this rejection should be reversed by the Board.

35 U.S.C. §112, first paragraph—Written Description

The disclosure as originally filed need not provide "*in haec verba* support for the claimed subject matter at issue," rather, the disclosure should convey to one skilled in the art that the inventor had possession of the invention at the time of filing. *Purdue Pharma L.P. v. Faulding, Inc.*, 230 F.3d 1320, 1323 (Fed. Cir. 2000). The Patent Office has the initial burden of presenting evidence or a reason why persons of ordinary skill in the art would not have

recognized such a description of the claimed invention in the original disclosure. See *In re Gosteli*, 872 F.2d 1008, 1012 (Fed. Cir. 1989).

D. Under 35 U.S.C. § 112, first paragraph, was it proper to reject claims 99, 101, 104-113, 116, 120, and 142-145 as allegedly containing new matter?

The final Office Action states that the specification and claims as originally filed provide support for a method step of allowing culture for a time sufficient to allow the antigen to bind to the dendritic cells "but not for the additional limitation of sufficient time to process and express the antigen."

Support in the present specification for each claim is discussed in the Summary of Claimed Subject Matter (section V) above and is also described in detail for the '612 priority application in the chart included as Appendix A. Specific support for the last step of claim 101 and 145 and the corresponding step of claim 120 are as follows:

> "Dendritic cells bind and modify antigens in a manner such that the modified antigen when presented on the surface of the dendritic cell can activate T-cells to participate in the eventual production of antibodies. The modification of antigens by dendritic cells may, for example, include fragmenting a protein to produce peptides which have regions which specifically are capable of activating T-cells." (page 5, lines 20-27)

> "The antigen-activated dendritic cells of the invention are produced by exposing antigen, *in vitro*, to the dendritic cells prepared according to the method of the invention. Dendritic cells are plated in culture dishes and exposed to antigen in a sufficient amount and for a sufficient period of time to allow the antigen to bind to the dendritic cells." (page 34, line 34 through page 35, line 3)

> "Cells should be exposed to antigen for sufficient time to allow antigens to be internalized and presented on the cell surface." (page 36, line 31-33)

> "Foreign and autoantigens are processed by the dendritic cells of the invention to retain their immunogenic form. The immunogenic form implies processing the antigen through fragmentation to produce a form of the antigen that can be recognized by and stimulate T cells." (page 34, lines 16-20)

Thus, the specification provides support for the relevant step of claim 101 (specifically, "culturing the dendritic cells *in vitro* in the presence of an antigen for a time sufficient to allow the antigen to bind to the dendritic cells, wherein the dendritic cells process the antigen to produce a modified antigen which is expressed by the dendritic cells"), claim 120 (specifically, "exposing the cells to antigen *in vitro*, wherein the dendritic cells process the antigen to produce

a modified antigen which is expressed by the dendritic cells), and 145 (specifically, "pulsing the dendritic cells with an antigen, wherein the dendritic cells process the antigen to produce a modified antigen which is expressed by the dendritic cells"; the term "pulsing" is synonymous with exposing the cell to a protein antigen, for example, as it is used in the Abstract of the Disclosure). While each independent claim (101, 120, and 145) includes slightly different terminology, the specification provides support for the terminology of each claim. Accordingly, the Board should reverse this rejection.

35 U.S.C. §112, second paragraph—Indefiniteness

35 U.S.C. §112, second paragraph is satisfied if "one skilled in the art would understand the bounds of the claim when read in light of the specification." *Miles Laboratories, Inc. v. Shandon, Inc.*, 997 F.2d 870, 875 (Fed. Cir. 1993). Claims meet the requirements of 35 U.S.C. §112, second paragraph, if "the claims, read in light of the specification, reasonably apprise those skilled in the art both of the utilization and scope of the invention, and if the language is as precise as the subject matter permits." *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1385 (Fed. Cir. 1986).

E. Under 35 U.S.C. §112, second paragraph, was it proper to reject claim 120 as allegedly indefinite?

The final Office Action alleges that claim 120 is indefinite because "it is unclear whether or not the actions of the claim are actually intended to be method steps. If so, then the steps must be separated and indented as is required of all method steps."

Claim 120 is as follows:

"An *in vitro* composition comprising mature dendritic cells expressing a modified antigen and derived from an *in vitro* culture of a population of proliferating dendritic cell precursor cells **by a method comprising culturing** dendritic cell precursor cells in a culture medium comprising GM-CSF at a concentration sufficient to promote the survival and proliferation of dendritic cell precursors; **serially subculturing** the proliferating dendritic cell precursors at intervals which provide for the continued proliferation of said dendritic cell precursors; **and exposing** the cells to antigen *in vitro*, wherein the dendritic cells process the antigen to produce a modified antigen which is expressed by the dendritic cells." (bold font added)

As shown by the terms placed in bold font, the claim recites that the dendritic cells are derived from a population of proliferating precursor cells "**by a method**" and then recites three clauses, each of which includes an active verb. The clauses are separated by semicolons. In view of the explicit statement in the claim regarding the use of a method and the proper use of active verbs and punctuation, Applicants' representative believes the claim meets the requirements for clarity under 35 U.S.C. § 112, second paragraph. Further, the grounds for the rejection—that the lack of indentation of the method steps renders the claim indefinite—does not appear to have any basis in the statute itself. Therefore, this rejection should be reversed by the Board.

CONCLUSION

For the reasons discussed above, the Examiner's rejections are improper and should be reversed by the Board. Appellants submit that the pending claims are in condition for allowance and earnestly solicit an early notice to that effect.

Respectfully submitted,

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VIII. CLAIMS APPENDIX

99. The pharmaceutical composition according to claim 116, wherein the dendritic cells express an amount of the modified antigen to provide between about 1 to 100 micrograms of the modified antigen in said pharmaceutical composition.

101. An *in vitro* composition comprising mature dendritic cells expressing modified antigen and derived from an *in vitro* culture of an enriched and expanded population of proliferating dendritic cell precursors by a method comprising:

providing a tissue source comprising dendritic cell precursors;

treating the tissue source comprising dendritic cell precursors to increase the proportion of dendritic cell precursors;

treating the tissue source to obtain a population of cells suitable for culture *in vitro*;

culturing the tissue source on a substrate in a culture medium comprising GM-CSF to obtain nonadherent cells and cell clusters;

subculturing the nonadherent cells and cell clusters to produce cell aggregates comprising proliferating dendritic cell precursors;

serially subculturing the cell aggregates one or more times to enrich the proportion of dendritic cell precursors; and

continuing to culture the dendritic cell precursors for a period of time to allow them to mature into mature dendritic cells;

culturing the dendritic cells *in vitro* in the presence of an antigen for a time sufficient to allow the antigen to bind to the dendritic cells, wherein the dendritic

cells process the antigen to produce a modified antigen which is expressed by the dendritic cells.

104. The composition according to claim 101, wherein the tissue source is blood.

105. The composition according to claim 101, wherein the tissue source is bone marrow.

106. The composition according to claim 101, wherein GM-CSF is present in the culture medium at a concentration of about 1-1000 U/ml.

107. The composition according to claim 104, wherein the concentration of GM-CSF in the culture medium is about 30-100 U/ml.

108. The composition according to claim 105, wherein the concentration of GM-CSF in the culture medium is about 500-1000 U/ml.

109. The composition according to claim 101, wherein the cell aggregates are blood derived and are subcultured from about one to five times.

110. The composition according to claim 101, wherein the cell aggregates are subcultured one to five times.

111. The composition according to claim 101, wherein the culture medium is selected from the group consisting of RPMI 1640, DMEM and α-MEM, and wherein the culture medium is supplemented with serum.

112. The composition according to claim 104, wherein the tissue source is treated to remove red blood cells.

113. The composition according to claim 105, wherein the tissue source is treated to remove B cells and granulocytes.

116. A pharmaceutical composition comprising a therapeutically effective amount of the composition according to claim 101.

120. An *in vitro* composition comprising mature dendritic cells expressing a modified antigen and derived from an *in vitro* culture of a population of proliferating dendritic cell precursor cells by a method comprising culturing dendritic cell precursor cells in a culture medium comprising GM-CSF at a concentration sufficient to promote the survival and proliferation of dendritic cell precursors; serially subculturing the proliferating dendritic cell precursors at intervals which provide for the continued proliferation of said dendritic cell precursors; and exposing the cells to antigen *in vitro*, wherein the dendritic cells process the antigen to produce a modified antigen which is expressed by the dendritic cells.

142. The composition according to claim 101, wherein the dendritic cell precursors are human.

143. The composition according to claim 142, wherein the dendritic cell precursors are obtained from blood.

144. The composition according to claim 142, wherein the dendritic cell precursors are obtained from bone marrow.

145. An *in vitro* composition comprising antigen-activated dendritic cells expressing modified antigens and derived from an *in vitro* culture of proliferating dendritic cell precursors by a method comprising:

a) providing a tissue source comprising dendritic cell precursors;

b) treating the tissue source to obtain a population of cells suitable for culture *in vitro*;

c) culturing the tissue source on a substrate in a culture medium comprising GM-CSF to obtain nonadherent cells and cell clusters;

d) subculturing the nonadherent cells and cell clusters to produce cell aggregates comprising proliferating dendritic cell precursors;

e) serially subculturing the cell aggregates one or more time to enrich the proportion of dendritic cell precursors;

f) continuing to culture the dendritic cell precursors for a period of time sufficient to allow them to mature into mature dendritic cells; and

g) pulsing the dendritic cells with an antigen, wherein the dendritic cells process the antigen to produce a modified antigen which is expressed by the dendritic cells.

IX.   EVIDENCE APPENDIX

1. Pancholi *et al.*, "Dendritic cells efficiently immunoselect mycobacterial-reactive T cells in human blood, including clonable antigen-reactive precursors," *Immunology* vol. 76, pp. 217-224 (1992).

This reference was previously made of record by the Examiner and is discussed on page 4 of the final Office Action. A copy is attached.

2. Inaba *et al.*, "Dendritic cells pulsed with protein antigens *in vitro* can prime antigen-specific, MHC-restricted T cells *in situ*," *J. Exp. Med.* vol. 172, pp. 631-640 (1990).

3. Steinman *et al.*, "The sensitization phase of T-cell-mediated immunity," *Ann. N.Y. Acad. Sci.* vol. 546, pp. 80-90 (1988).

4. Markowicz and Engleman, "Granulocyte-macrophage colony-stimulating factor promotes differentiation and survival of human peripheral blood dendritic cells *in vitro*," *J. Clin. Invest.* vol. 85, pp. 955-961 (1990).

References "2," "3," and "4" are discussed on page 5 of the final Office Action. References "2" and "4" were cited in an IDS in 2001, and references "3" and "4" were made of record by the Examiner in 2009. Copies are attached.

Dendritic cells efficiently immunoselect mycobacterial-reactive T cells in human blood, including clonable antigen-reactive precursors

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SUMMARY

Given the persistence of tuberculosis throughout the world, the delineation of mechanisms that lead to protective immunity to Mycobacterium tuberculosis is important. We have evaluated the presenting function of human dendritic cells for mycobacterial antigens. since these antigen-presenting cells (APC) are particularly effective in initiating antigen-specific T-cell responses. Dendritic cells from blood prove to be active APC for mycobacteria-specific proliferative responses by CD4⁺ T cells from bacillus Calmette-Guérin (BCG)-vaccinated individuals. In the first 24–48 hr of the response, dendritic cells that have been pulsed with mycobacterial antigens. including live BCG. effectively bind T cells forming discrete cell clusters. The clusters represent about 1% of the applied T cells. Clusters are highly enriched in mycobacterial reactivity while the non-clusters are depleted. Clustered T cells can be used as a starting point to expand antigen-specific cell lines. Mitogen and allogeneic feeder cells were used as APC to expand the mycobacterial-reactive lines. because the antigen-specific T cells had been preselected by virtue of their binding to antigen-pulsed dendritic cells. We discuss the advantages of obtaining antigen-reactive T cells by using dendritic cells as immunoadsorbents. These lines should help delineate the range of mycobacterial antigens and T-cell responses that participate in host responses to mycobacteria.

INTRODUCTION

The majority of individuals exposed to or infected with pathogenic mycobacteria develop effective immunity that is based on cellular immune responses. T-helper/inducer lymphocytes are of particular importance[1,2] as indicated by the resurgence of tuberculosis during human immunodeficiency virus (HIV)-1 infection.[3] An essential step in the activation of CD4⁺ T-helper cells to complex antigens like mycobacteria is the presentation of antigen fragments in conjunction with class II molecules of the major histocompatibility complex (MHC). Macrophages can serve as antigen-presenting cells (APC). but it is of interest that macrophages are also hosts for virulent mycobacterial replication.[4,5] Therefore a role for dendritic cells which are poorly phagocytic but specialized APC for acquiring antigen in vivo and for inducing T-cell-mediated immunity.[6-9] may be central in understanding protective mechanisms. There is but a single report in the mouse[10] that dendritic cells can present mycobacteria.

An important property of dendritic cells which sets them apart from other APC that are resident in lymphoid tissues, is their capacity to form large aggregates with antigen-specific T cells in primary tissue culture systems. This has been studied most in the allogenic mixed leucocyte reaction (MLR) where it is observed that mouse,[11] rat[12] and human[13] dendritic cells efficiently cluster CD4⁺ T cells that are specific for the presented transplantation antigen. Alloreactivity is enriched in cells derived from the clusters and depleted from cells in the non-clustered population.[13,14] This separation of antigen-reactive and non-reactive CD4⁺ T cells is evident at the level of clonable precursors as well. i.e. one can use the clusters that develop in the MLR as an enriched source of antigen-specific T-cell lines.[14] Because of the immunoselection imparted by the clustering step. the bound antigen-specific T cells can be efficiently and simply expanded with mitogen-treated. MHC mismatched feeders. Antigen-specific T cells also cluster with dendritic cells during murine T-cell responses to soluble protein antigens.[15,16] but similar studies have not been performed with complex microbes like the mycobacteria.

We have now performed such experiments in the mycobacterial system in man and show that human dendritic cells. like their murine counterparts. present mycobacterial antigens to CD4⁺ T cells from immunized donors. namely individuals vaccinated with bacillus Calmette Guérin (BCG). In addition. dendritic cells that had been pulsed with live BCG cluster or bind most mycobacteria-reactive CD4⁺ T cells. The clusters then serve as a highly enriched source of precursors that can be

Abbreviations: APC. antigen-presenting cells; MHC. major histocompatibility complex; MLR. mixed leucocyte reaction.

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expanded with mitogen as mycobacterial reactive T-cell lines. We discuss the use of this approach in generating T-cell lines that recognize antigens being carried by APC, including APC from patients with tuberculosis and other clinical disorders.

MATERIALS AND METHODS

Culture medium
RPMI-1640 (Gibco, Grand Island, NY) supplemented with 5% human AB⁻ serum and Ampicillin (Sigma 100 µg/ml; Sigma Chemical Co. St Louis, MO) was used throughout.

Human subjects
Blood was obtained from donors with informed consent. The subjects, vaccinated with BCG, were categorized into 'high' and 'low' responders depending on the ability of their blood mononuclear cells to respond to mycobacterial antigens (stimulation indices of > 100 versus < 10). Eight such 'high' responders were used for these studies.

Mycobacterial antigens
(a) *Mycobacterium bovis*. The BCG Pasteur strain 1011 was obtained from the Trudeau Institute (Saranac Lake, NY) and was grown at 37 in Proskauer-Beck medium containing 0·05% Tween-80 as shake cultures. Cultures were harvested in their logarithmic phase and frozen at −180 at 3×10^8 colony-forming units (CFU)/ml until use. Since the cultures of live BCG would also contain some dead bacteria in different stages of autolysis, CFU were determined after plating serial 10-fold dilutions of *M. bovis* BCG cultures on Middlebrook and Cohn 7H10 plates (BBL cat. no. 21174) in triplicate at 37 for 15–20 days.
(b) *M. tuberculosis* acetone precipated fraction (APF). APF was prepared as previously described.[17] Before use, samples were brought to the desired concentration in medium with 5% AB⁻ serum and sterilized.

Preparation of CD4⁺ T cells
T-cell-enriched populations were isolated by rosetting blood mononuclear cells with neuraminidase-treated erythrocytes (E). The ER⁺ fraction was the source of CD4⁺ T cells as described previously.[14] The resulting CD4⁺ T cells were > 95% pure as assessed by analysis on a FACScan Cytofluorograph with the Leu-3 monoclonal antibody (mAb) (Becton Dickinson, Mountain View, CA).

Preparation of APC
The ER cells were the source of APC. Monocytes were first isolated by adherence to tissue culture plates (Falcon Labware, Fisher Scientific, Pittsburg, PA; cat. no. 3003). After 1 hr, the non-adherent cells were collected. The adherent cells were washed two more times with warm RPMI, to dislodge loosely adherent cells, and then the firmly adherent monocytes were dislodged with cold Hanks' or phosphate-buffered saline (without calcium or magnesium). The monocytes were > 90% pure as judged by staining with Leu-M3 (anti-CD14) mAb. The ER⁻ non-adherent fraction (T-cell and monocyte depleted), was depleted of residual monocytes by panning twice on Ig-coated Petri dishes. This provided populations that were about 75% B cells, 5–10% natural killer (NK) cells and 1–3% dendritic cells.[13] These cells were layered onto Metrizamide gradients and spun at 650 *g* at room temperature for 10 min.[18] The low-density,

dendritic cell-enriched fraction was collected, washed twice in RPMI-1640 containing 10% foetal calf serum and sodium chloride (0·23–0·14%) to gradually decrease the osmolarity, and resuspended in RPMI containing 5% human AB⁻ serum. The low-density cells were 50–70% dendritic cells with some contaminating NK (10–15%), B (18–25%) and T (2–5%) cells.[18] The metrizamide 'sinkers' or pellets were used as APC and consisted primarily of B cells.

Further purification of dendritic cells and macrophages by cell sorting
Partially enriched dendritic cells obtained from the low-density fraction of metrizamide gradients were further purified by sorting with a FACStar Plus instrument (Becton Dickinson, Immunocytometry Systems, San Jose, CA) as described previously.[18] For dendritic cell sorting, the low-density fraction was incubated with phycoerythrin (PE)-conjugated monoclonal antibodies (Becton Dickinson) specific to cell surface markers expressed by non-dendritic cells, i.e. CD14, CD56, CD19 and CD16. For macrophage sorting, plastic adherent cells were incubated with the above-mentioned mAb *except* that anti-CD14 was omitted. The B-cell-enriched APC population obtained from the metrizamide pellet fraction was not sorted, since it was adequately free of other cell types. The purity of the different APC populations was determined by staining with mAb to CD14, CD56, CD19, CD16 and HLA-DR (Becton Dickinson, Mountain View, CA). Note that dendritic cells are very rich in HLA-DR but negative for macrophage (CD14) and B cell (CD16) markers (Fig. 1).

Comparison of the stimulatory capacity of different APC in presenting mycobacterial antigens to bulk T-cell cultures
ER cells were pulsed with live *M. bovis* BCG for 12–14 hr (10 organisms/white cell) after which the different APC populations (above) were prepared. Graded doses of these APC were cultured with 1.5×10^5 bulk CD4⁺ T cells at APC:T cell ratios of 1:5 to 1:100 in flat 96-well microtitre plates for a period of 5–6 days. The cultures were pulsed with 1 µCi [³H]thymidine (specific activity 20 Ci/mmol; NEN Research Products, Wilmington, DE) for the last 12–16 hr. In parallel, antigen non-pulsed APC populations were added to bulk CD4⁺ to assess background proliferation (syngeneic MLR).

Antigen pulsing of monocyte-depleted stimulator cells and the formation of dendritic cell-T cell clusters
ER cells were pulsed with live *M. bovis* BCG or live *M. tuberculosis* H37Ra overnight, or with mycobacterial APF for 2 hr, washed twice and then depleted of monocytes by FcR panning. 1.5×10^6 CD4⁺ T cells were stimulated with 3×10^5 syngeneic APC that had been pulsed with antigen (APF or live mycobacteria) in 1 ml RPMI-1640 containing 5% human serum in flat-bottom 16-mm well plates (Costar, Cambridge, MA) for 36–48 hr. During this time, clusters of dendritic cells and responding T cells developed.[14] To isolate clusters, the cultures were gently resuspended and applied to 30% human serum gradients. After 2 hr, the clustered T cells sedimented to the bottom while the non-clustered T cells were harvested from the top 2–3 ml (if 5 ml is initially applied to the gradients). Clusters were then cultured in 5% AB⁺ medium in 16-mm wells for an additional hour to allow loosely adherent cells to dislodge. The
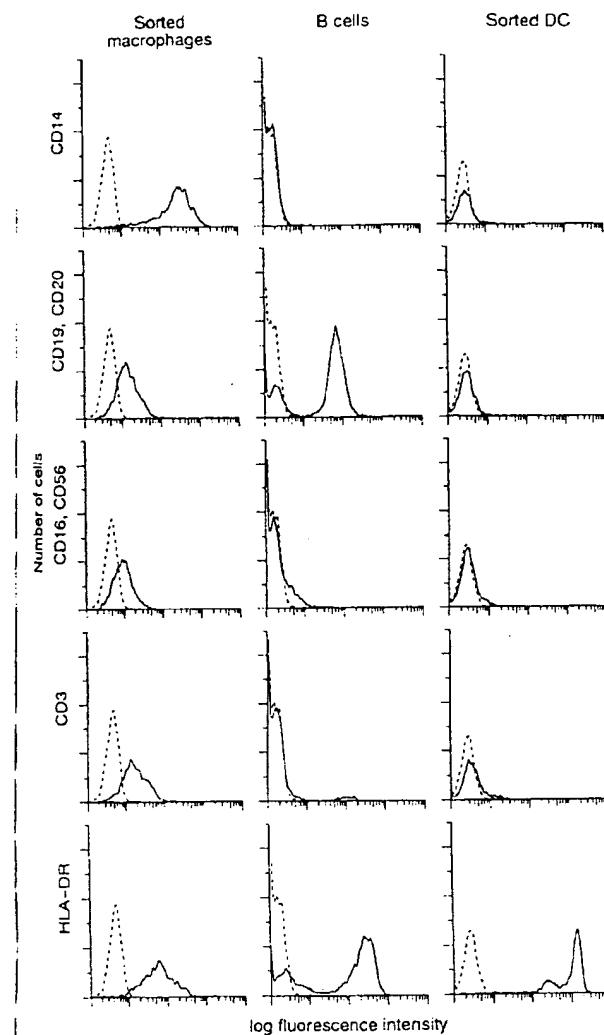
Figure 1. Surface markers of highly enriched monocytes, dendritic cells and B cells. Partially enriched dendritic cells, obtained from the low-density fraction of metrizamide gradients were stained with PE-conjugated anti-CD3, CD14, CD16, CD19 and CD56. Macrophages, obtained after plastic adherence of ER⁻ cells, were stained with the same mAb except for anti-CD14. The stained cell populations were then sorted on FACStar plus to provide fluorescent negative dendritic cells and monocytes respectively. The metrizamide sinkers or pellet were used as a source of B cells and were not sorted. The purity of the different APC populations was determined by staining with PE conjugates of CD14, CD19, CD56 and HLA-DR. Note that the dendritic cells lack CD14 (monocyte), CD19 20 (B cells), CD16 56 (NK cells) and CD3 (T cells) but have much higher levels of HLA-DR than monocytes or B cells.

cells were reapplied to serum gradients to again sediment the clusters. The non-clustered T cells were also reapplied to serum gradients for 1 hr to ensure removal of small clusters.

Development of T-cell lines

T-cell lines were generated from clustered, non-clustered and bulk (unseparated) fractions, the clusters being disrupted by gentle pipetting. Varying numbers of cells (1–1000) from the above fractions were seeded in limiting dilution in round-bottom 96-well microtitre plates to which 10³ irradiated (1500 rads from ¹³⁷Cs source) stimulators were added. Two types of APC (ER⁺) were used to expand T cells: syngeneic ER cells pulsed with mycobacterial antigen, or mitogen sodium periodate-treated allogeneic ER cells as described recently.[14] Parallel control plates were seeded with T cells in the absence of any stimulator cells, or in the presence of antigen only. Likewise, stimulator (ER) cells (periodate modified or unmodified) were added at 10³ per well either alone or in presence of only antigen but no T cells. Purified interleukin-2 (IL-2) (15% v/v; Pharmacia ENI, Silver Springs, MD) was added as growth factor to all the plates. T-cell lines became apparent within 7–10 days and were then transferred to 16-mm macrowells for further expansion with 10⁶ periodate-treated allogeneic APC and IL-2.

Determination of antigen specificity of clustered T cells

(a) *Bulk cultures of clustered and non-clustered T cells.* Clustered as well as non-clustered T cells were collected as described after 2 days of APC–T cell co-culture. The cells were then cultured in 5% AB⁺ medium for 4–5 days during which time the clusters proliferated, released blasts, and began to rest down. Antigen specificity was determined by culturing graded doses (3 × 10³ to 2 × 10⁵) of clustered, non-clustered or bulk CD4⁺ (unseparated) T cells with 10⁵ syngeneic ER cells in the presence of antigen (1 μg/ml BCG or 5 μg/ml APF). Duplicate plates were set up. One set of cultures was pulsed at Day 2 (to detect a rapid secondary response) and the other set at Days 5–6 with 1 μCi [³H]thymidine for 12–14 hr. Tetanus toxoid (T. Tox) added at 2 lf/ml was included as a control antigen to assess the mycobacterial specificity of the response.

(b) *T-cell lines derived from clusters and non-clusters* The specificity of the cell lines generated from clustered, non-clustered and /or bulk CD4⁺ T-cell fractions was determined either *immediately* after the generation of the cell lines or after their expansion into macrowells. When tested immediately, the cell lines were washed free of residual IL-2 by spinning plates at 750 g for 2 min and washing with RPMI-1640. The cell line(s) were then resuspended well in 300 μl of 5% serum containing media. This was divided into seven parts. One part was used to expand with periodate-modified APC and IL-2 (in case the line proved to be antigen reactive and in need for further study). The other six parts (about 5–10,000 cells each) were used for rechallenge with APC either in presence or absence of mycobacterial antigen. Alternatively, 2 × 10³ T cells from expanded cell lines were cultured with 10⁵ irradiated ER cells from syngeneic donors in the presence of specific mycobacterial antigen (APF, 5 μg/ml) and /or irrelevant antigen tetanus toxoid (2 lf/ml). The assay was performed in a total volume of 200 μl in flat-bottomed microtitre plates (Costar, Cambridge, MA). After 48 hr, cultures were pulsed with 1 μCi of [³H]thymidine for 14–16 hr. The data are expressed as stimulation indices, i.e. c.p.m. with APC plus APF or T.Tox/c.p.m. with APC alone.

RESULTS

### Presentation of mycobacterial antigens by human blood dendritic cells and other APC

Dendritic cells, monocytes and B cells were compared for their capacity to elicit a proliferative response from CD4⁺ T cells
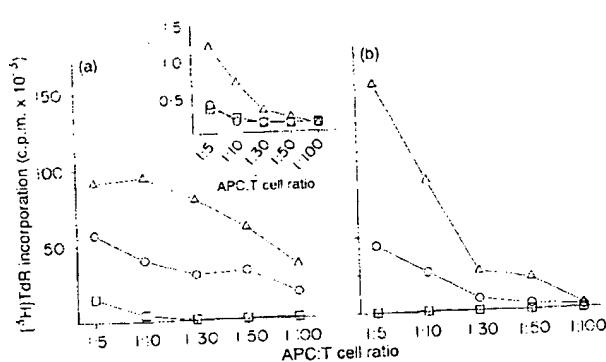
Figure 2. Dendritic cells efficiently present mycobacterial antigens to primed CD4$^+$ T cells. T-cell-depleted. blood mononuclear cells from BCG-vaccinated donors were pulsed with live *M. boris* BCG for 14–16 hr. Different APC populations (macrophages, dendritic cells and B cells) were prepared as described in Materials and Methods. APC that were either partially purified (a). or purified after cell sorting (b). were added in graded doses to 1.5×10^5 CD4$^+$ T cells (1:5–1:100). Proliferative responses were determined on Days 6–7 after pulsing with [^{3}H]TdR for 16–18 hr. With uninfected APC populations. weak background proliferative responses (syngeneic MLR) were observed (see inset). Dendritic cell:T cell (△). macrophage:T cell (○): B cell:T cell (□).

obtained from BCG vaccinated donors. To prepare antigen-pulsed APC. T-depleted (ER) cells were cultured with whole live *M. boris* BCG inoculum for 12–14 hr. and then different populations of APC were prepared (see Materials and Meth-
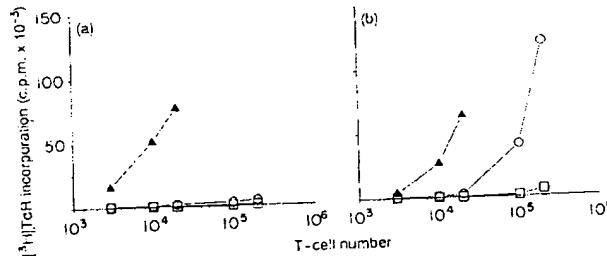
Figure 3. Mycobacteria-reactive T cells are clustered by antigen-pulsed dendritic cells. Monocyte-depleted APC populations from a BCG-vaccinated donor were pulsed with live *M. boris* BCG and cultured with CD4$^+$ T cells (see Materials and Methods). Forty eight hours later. APC-T cell aggregates (clusters) were separated from non-clustered T cells over serum gradients. These fractions. as well as unseparated bulk T cells. were returned to culture for 4–5 days during which time the clustered T cells proliferated and began to rest down. Clustered (▲). non-clustered (□) and bulk (○) T cells were then rechallenged with 10^5 syngeneic APC that had been pulsed or not pulsed with live mycobacteria. Proliferation of graded doses of T cells was measured at Day 2 (a) and at Day 6 (b). Antigen-specific responses could be detected with clustered T cells as early as Day 2. With bulk CD4$^+$ T cells (which are comprised of a mixture of antigen-specific as well as antigen-non-specific T cells) responses could be detected by Day 6. The non-clustered T cells. however. were depleted of antigen reactivity both on Day 2 and on Day 6. No responses were seen in the absence of antigen (not shown).

Table 1. Yield of cell lines from clustered, non-clustered and bulk T cells. CD4$^+$ T cells from BCG vaccines were cultured with mycobacterial CD4$^+$ T cells from BCG vaccines were cultured with mycobacterial antigen-pulsed ER$^+$FcR$^+$ cells. The APC were pulsed with different forms of mycobacteria in the three experiments shown (a–c). After 2 days. clustered cells were separated from non-clustered T cells. Graded doses of these cells were expanded as cell lines either in the presence of IL-2 and no APC. syngeneic APC plus antigen [*Mycobacterium* APF. H37Ra or BCG in (a). (b) and (c) respectively] or sodium periodate-treated allogeneic APC. In (c) lines derived from clusters and non-clusters were directly compared with bulk unseparated T cells. Cell lines were visualized 7–10 days later. Results are expressed as yields of cell lines per total number of wells seeded. Antigen reactivity of lines is illustrated in Figs 4 and 5

	Yield of cell lines in the presence of		
	No APC	Syngeneic APC plus antigen	Sodium periodate-modified APC
(a) After pulsing with mycobacterial acetone precipitate fraction			
Clusters			
1 cell/well	0.48	5.96	9/60
10 cell/well	6.48	46.96	35.60
Non-clusters			
1 cell/well	0/48	0.96	1/60
10 cell/well	0.48	4/96	27.96
(b) After pulsing with live *Mycobacterium tuberculosis* H37Ra			
Clusters			
1 cell/well	0/48	10/96	10/96
3 cell/well	1.48	18/96	23/96
10 cell/well	6/48	47.96	64.96
Non-clusters			
1 cell/well	0/48	0/96	7.96
3 cell/well	0.48	0/96	21.96
10 cell/well	0/48	0.96	54/96
(c) After pulsing with live *Mycobacterium boris* BCG			
Bulk			
10 cell/well	0/48	2.96	8.96
100 cell/well	0/48	12.96	45.96
1000 cell/well	0.48	62.96	95.96
Clusters			
3 cell/well	1/48	20.96	21.96
10 cell/well	3/48	45.96	42.96
100 cell/well	39/48	77.96	91.96
Non-clusters			
10 cell/well	0/48	5.96	11/96
100 cell/well	0.48	8.96	43.96
1000 cell/well	0/48	18.96	93.96

ods). The APC were added in graded doses to CD4$^+$ T cells and proliferative responses determined on Days 6–7. As is evident from Fig. 2a (representative of three such experiments), the antigen-pulsed. dendritic cell-enriched fraction presented mycobacteria. Dendritic cells were more active than monocytes. while B cells were inactive. Similar results were obtained with the acetone precipitate fraction of H37Ra (data not shown). To verify that the dendritic cells were in fact the active APC. dendritic cells were purified (> 95%) by cell sorting.[8] The sorted dendritic cells were several times better than sorted monocytes
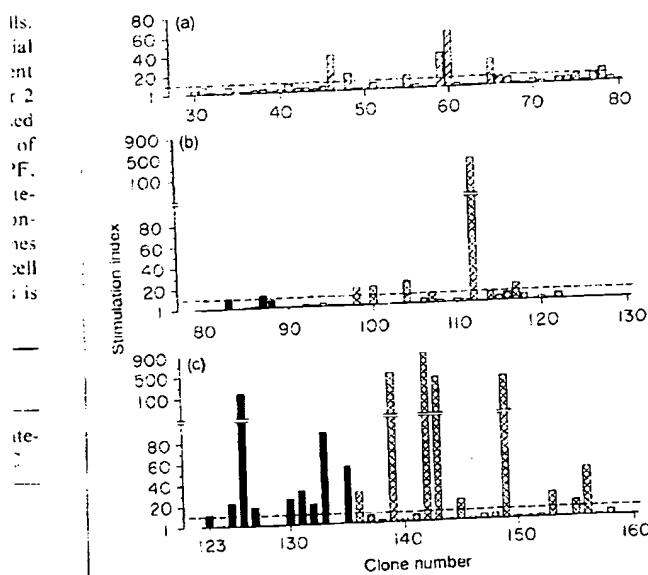
Figure 4. T cells selected in the APC–T cell clusters are an enriched source of mycobacterial-specific T-cell lines. T cells from a BCG-vaccinated donor were cultured with *M. bovis* BCG pulsed APC that had been T-cell and monocyte depleted. After 48 hr, clustered T cells were separated from non-clustered cells as described in Materials and Methods. T-cell lines were then generated from graded numbers of bulk, cluster and non-cluster T cells in the presence of either syngeneic APC plus antigen or in the presence of mitogen (sodium periodate-modified allogeneic APC). The lines were evident at about 7–10 days after seeding and were immediately checked for antigen specificity to mycobacterial APF. Results are expressed in stimulation indices (SI) where SI=[³H]TdR uptake of cell line in the presence of *M. tuberculosis* APF-pulsed PBMC divided by [³H]TdR uptake of cell line with PBMC alone. Antigen responsive lines (stimulation indices of > 10) were derived from bulk T cells (a) after plating at 1000 cells/well (▨); 100 cells/well (▢). In contrast, antigen-specific T-cell lines from clustered T cells were obtained after plating either 3 cells/well (■) or 10 cells/well (▢) (b, c). Expansion of clustered T cells with mitogen (c) generated larger numbers of antigen-specific cell lines. Non-clustered T-cell lines did not respond to antigen (not shown).

in stimulating bulk T cells (Fig. 2b). Control experiments, where the APC fractions were not pulsed with antigen, showed very little stimulatory activity [syngeneic MLR; Fig. 2 inset].

Antigen-pulsed dendritic cells cluster mycobacterial-reactive T cells

In the standard APC assay system above, one does not directly assess the efficacy with which APC bind antigen-specific T cells. To approach this issue we extended prior work in the primary MLR system in which we showed that dendritic cells cluster antigen-specific T cells from bulk T-cell cultures.[14] This feature is distinctive for dendritic cells since B cells, NK cells or macrophages do not enter the clusters. We tested if antigen-pulsed dendritic cells would retrieve most mycobacterial-reactive T cells from bulk CD4+ cultures. T-depleted (ER+) cells from BCG-primed individuals were again pulsed with mycobacterial antigens, i.e. inocula containing either whole live *M. bovis*
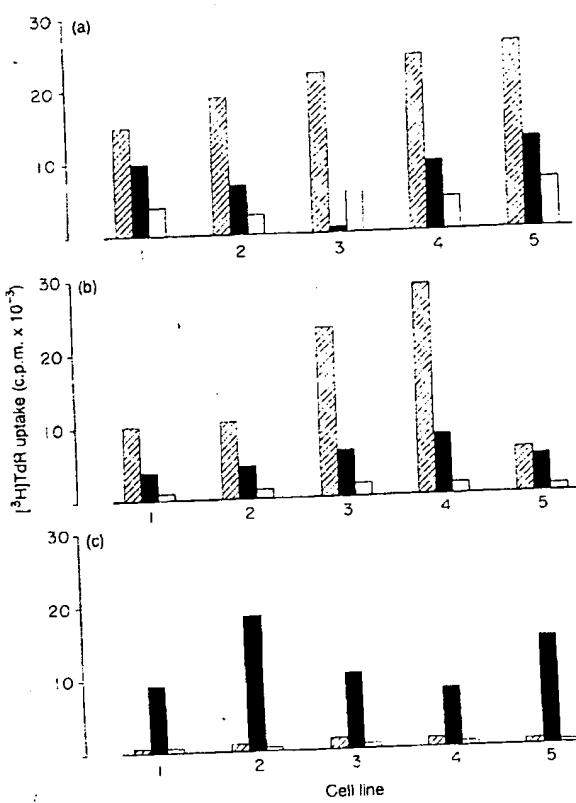

Figure 5. Dendritic cell–T cell clusters are an enriched source of mycobacterial responsive T-cell lines that maintain antigen specificity for 5–6 months. T-cell-depleted mononuclear cells were pulsed for 2 hr with mycobacterial APF at 5 µg/ml. Partially enriched dendritic cells were prepared by depleting monocyte and T cells. These were co-cultured with CD4+ T cells for 36–48 hr after which clusters were separated from non-clustered T cells. Graded doses (1–10) of clustered T cells were expanded with IL-2 plus syngeneic APC pulsed with APF (a) and/or mitogen (b). Likewise, non-clustered T cells were expanded with mitogen sodium periodate-treated allogeneic APC (c). The lines were repetitively stimulated for a period of 5–6 months during which time individual lines were tested for antigen specificity to either *M. tuberculosis* acetone precipitable fraction versus T. Tox in presence of syngeneic APC. The data are expressed as [³H]TdR incorporation (c.p.m.) with APC plus APF (■) or T. Tox (▢). C.p.m. in the presence of APC alone were < 500. As is evident, the cluster-derived lines were found to respond preferentially to *M. tuberculosis* APF versus T. Tox. This was true even in panels where the cluster-derived lines were maintained on mitogen-treated APC and not specific antigen. The non-cluster-derived lines lacked reactivity to mycobacterial APF but responded to sodium periodate-treated APC (■) which represented a mitogen control.

BCG, or *M. tuberculosis* H37Ra or mycobacterial antigen APF and then depleted of Fc receptor-bearing (FcR+) monocytes by panning on γ-globulin-coated dishes. The ER–FcR– population was co-cultured with bulk CD4+ T cells at a ratio of 1:5 for 36–48 hr. As previously described for the MLR,[13,14] the use of monocyte-depleted APC allowed the rapid development of large dendritic cell–T cell clusters (containing about 1% of the total cells in the culture) that were easily isolated on serum gradients. The APC in the clusters had the same phenotype as the dendritic

cells, i.e. rich in HLA-DR but negative for CD14, CD3 and CD19 markers.[14]

Antigen reactivity was found to be confined to, and highly enriched in, T cells derived from the clustered fraction (Fig. 3). When clustered T cells were rechallenged with antigen plus syngeneic APC, strong secondary responses occurred even with small numbers (3×10^3) of cluster-derived cells (Day 2 [³H]-TdR uptake; Fig 3a). Non-clustered T cells did not respond at Day 2 or even after 6 days of secondary challenge (Fig. 3b). Bulk (unseparated) T cells showed the expected recall response at Day 6 (Fig. 3b). The response was specific to mycobacterial antigens since no response to the irrelevant tetanus toxoid antigen was noted in clustered T cells (data not shown). Thus dendritic cell–T cell clusters are a highly enriched source of antigen-reactive T cells, and most mycobacterial-reactive CD4⁺ T cells are aggregated.

Mycobacterial antigen-pulsed dendritic cell–T cell clusters are an enriched source of T cells that can be expanded with APC and IL-2

We next attempted to clone T cells from clusters, non-clusters and unseparated CD4⁺ T cells derived from BCG-vaccinated donors. Cloning was performed by seeding limiting numbers (1 1000) of cells in round-bottom wells of 96-well microtitre plates. IL-2 was added. As feeder cells, we used either syngeneic APC plus mycobacterial antigen or mitogen (sodium periodate-treated APC). These limiting dilution studies allowed us to compare the frequencies of generating antigen-specific T-cell lines (see below) from the above-mentioned T-cell fractions. After 7–10 days, growth of the T-cell lines was evident as described.[14,19] Different mycobacterial antigens (APF versus live mycobacteria) were assessed (Table 1).

A substantial number of cell lines developed from clustered T cells both in the presence of syngeneic APC plus antigen or in the presence of mitogen (sodium periodate-treated APC; Table 1). In the absence of mitogen, non-clustered T cells generated very few cell lines confirming that few antigen-reactive cells were present in this fraction. With mitogen, however, large numbers of cell lines were obtained (Table 1a,b). In the experiment where live *M. bovis* BCG was used to pulse the APC population prior to clustering, the frequency of cell lines generated from clusters and non-clusters was compared to bulk unseparated T cells (Table 1c). Note that when infected syngeneic APC were used to expand the lines, bulk T cells had a much higher incidence of antigen-dependent cell lines relative to non-clustered T cells. For the bulk cultures, limiting dilution, i.e 66% of the wells being positive, occurred at about 1000 CD4⁺ T cells/well (Table 1c). Since we were limited in the number of APC from donors, we used 10,000 T-cell-depleted cells which have about 100 dendritic cells. Prior work has shown that optimal cloning efficiency in a mitogen system requires 1000 dendritic cells.[19] Both non-cluster and bulk T cells gave a similar number of cell lines when mitogen was used with APC. The clustered T cells, however, were highly enriched in antigen-inducible cell lines. e.g. 10 clustered cells gave more antigen-inducible lines than 100 bulk T cells (45/96 wells versus 12/96). Even in the absence of further APC, a sizeable number of cell lines developed from clustered T cells seeded at 100 cells per well (Table 1c). Most likely, this was due to the carry-over of antigen-pulsed dendritic cells in the 100-cell inoculum of dendritic cell–T cell clusters, since dendritic cells represent 10–20% of the clustered cells.

Antigen specificity is confined to cell lines derived from dendritic cell–T cell clusters and not to cell lines obtained from non-clustered T cells

A large number of the cell lines generated in the experiments above (20–30 per variable) were then tested for reactivity to an acetone extract of mycobacteria. As is evident from Fig. 4a, bulk T cells that had been derived initially with syngeneic APC plus antigen showed some strongly antigen reactive lines. 7 45 wells had stimulation indices of > 10. The other 38,45 wells either were in a stage where it was difficult to respond well to antigen, or more likely, the initial growth in the well was due to factors from infected APC plus exogenous IL-2. T-cell lines grown from the non-clustered fraction with periodate-modified APC had no antigen-reactive lines (not shown). For clustered T cells that had been expanded with infected syngeneic APC, 7/40 lines had stimulation indices of > 10 (Fig. 4b). When sodium periodate-modified APC were used, almost half the lines tested were antigen reactive, including most of the lines that had been derived from just three clustered cells/well (Fig. 4 c). Clearly, the use of mitogen leads to a greater yield of cell lines that are antigen reactive. The antigen reactivity observed in the initial screen persisted upon subsequent growth and rechallenge (data not shown). The cell lines derived from APC T cell clusters in the above manner were primarily CD3⁺ CD4⁺ CD8 TcR $\alpha\beta^+$ in phenotype, although we did observe 1-2% $\gamma\delta$ T-cell lines (data not shown).

We also perpetuated cell lines generated from the cluster and non-cluster T cells after pulsing with mycobacterial APF, either in the presence of syngeneic APC plus angtigen or in the presence of periodate-treated allogeneic APC *long term*. Cell lines obtained from the clustered T-cell fraction in the above manner maintained antigen specificity to mycobacterial APF even when tested 5–6 months after successive restimulations (Fig. 5a, b). No reactivity to T. Tox control antigen was seen. Cell lines derived from the non-cluster T-cell fraction lacked reactivity to APF but proliferated to mitogen (periodate modified APC; Fig. 5). These findings confirm that antigen reactivity is highly enriched in the initial T-cell-clustered population. Furthermore, antigen specificity can be maintained long term when cell lines are obtained from this population.

DISCUSSION

Mycobacteria induce both cell-mediated and humoral immune responses. Protective immunity to mycobacterial infections is T-cell mediated,[20,22] a fact that has been further emphasized by the high incidence of miliary tuberculosis and atypical mycobacterial infections in acquired immune deficiency syndrome (AIDS).[3] Antigen-presenting cells play a major role in initiating immune responses and activating T cells. Macrophages have classically been ascribed a dominant role in antigen presentation during mycobacterial infections, and the subsequent macrophage-T lymphocyte interaction has been suggested to be important for controlling microbial growth.[23] Clinical tuberculosis, in effect, represents a failure of the immune system to eradicate mycobacteria within phagocytes. The role of human dendritic cells in presenting mycobacterial antigens has not been determined, presumably because enriched populations have become readily accessible from human blood only recently.[18] We felt it important to pursue the role of dendritic cells in mycobacterial

responses, since dendritic cells are specialized for inducing T-cell immunity to several other antigens, including antigen responses in whole animals.[9,24]

Initially, we compared the activity of different cell types (macrophages, dendritic cells and B cells) in presenting mycobacterial antigens to primed T cells obtained from BCG-sensitized donors. These individuals demonstrate recall responses to antigens of mycobacterial origin and therefore serve as a prototype for studying mycobacterial immune responses *in vitro*. Amongst different APC populations, purified dendritic cells presented both APF (data not shown) as well as live *M. bovis* BCG (Fig. 2) antigens to primed T cells. Dendritic cell populations were two- to fivefold better than macrophages, while B cells were completely inactive. These observations are intriguing, because virtually every cell in the monocyte preparation can be infected with many mycobacteria (as assessed by acid fast staining), while < 5% of the dendritic cells had only one- and rarely two-cell-associated organisms (data not shown).

The ability of human blood dendritic cells to present mycobacterial antigens to T cells in spite of poor phagocytic capacity may be explained in two ways. Either the few dendritic cells with a cell-associated organism are doing all the presenting, in which case, these dendritic cells are remarkably potent APC. Alternatively, the dendritic cells may acquire peptide fragments by some other means, e.g., regurgitation of peptides from the macrophage, or uptake of soluble mycobacterial antigens during the original antigen pulse or infection. Studies are currently underway to test if mycobacterial antigens can be transferred from macrophages to dendritic cells.

There are few studies of the capacity of dendritic cells to present microbial antigens. In humans, dendritic cells are extremely efficient presenters of staphylococcal enterotoxins (superantigens). They are of the order of 10–50-fold more efficient than B cells or macrophages.[25] In the mouse, several viruses can be presented by dendritic cells.[26–28] and in concordance with the present study a single publication available concluded that dendritic cells present mycobacterial antigens, soluble as well as particulate, as efficiently or more efficiently than macrophages.[10]

The main goal of the present investigation was to determine if antigen-pulsed blood dendritic cells could be used to retrieve mycobacterial-reactive T cells from bulk T-cell cultures. We observed that when dendritic cells were pulsed with mycobacterial antigens and then mixed with CD4⁺ T cells from BCG-vaccinated donors, large clusters formed. Most of the antigen-reactive T cells proved to bind to the dendritic cells (Fig. 3). These clusters represented an enriched source of antigen-reactive T-cell precursors that were then readily expanded as cell lines.

This approach for obtaining antigen-reactive cell lines has several advantages over current standard cloning protocols where one stimulates T cells with antigen and then adds IL-2 to expand responsive blasts prior to cloning under limiting dilution. (a) The generation of dendritic cell T cell clusters is simple to set up. The dendritic cells need only to be partially enriched and it takes 2 days to have T cells to clone as compared to 1–3 weeks if one first expands with antigen plus IL-2 in traditional systems. (b) Since dendritic cells are acting as immunoadsorbents, mycobacteria-specific cell lines derived from clustered T cells can be perpetuated by mitogen in the absence of specific mycobacterial antigen or even MHC-specific APC (Figs 4, 5).

This reduces the need for specific *donor* blood for long-term perpetuation, and it appears that mitogen-treated APC are more efficient at expanding the lines (Fig. 4). (c) As yet, there is no evidence that standard methods expand most mycobacterial-reactive T cells, whereas our results show that most antigen-specific CD4⁺ T cells cluster with dendritic cells (Fig. 3). (d) A considerable enrichment in the frequency of generating antigen-specific T-cell lines was observed with the dendritic cell clustering approach as compared to cloning with bulk (unseparated) T cells (Table 1c). Also non-clustered T cells were depleted of reactivity and only yielded lines in the presence of polyclonal mitogen. By starting with dendritic cell–T cell clusters, one can expand lines with just one to three cells per well (Table 1; Fig. 4). These lines are likely to be clonal given the fact that few T cells and limiting dilution conditions were used. (e) Because standard cloning procedures entail a good deal of cell growth *prior* to cloning, it is possible that one selects out cells that grow well or that recognize antigens that are processed well *in vitro*. T cells that are selected by binding to antigen-pulsed APC on the other hand may provide a wider spectrum of T cells that respond to mycobacteria.

The T-cell clones that have been developed from tuberculosis/leprosy patients have been raised in the presence of *exogenous* mycobacterial antigens. These recognize 12,000, 19,000, 65,000 and 71,000 MW antigens, the latter being heat-shock proteins.[29,30] Many of these antigens were defined by monoclonal antibodies to antigens recognized as being immunodominant for B cells.[31] Since antibodies are unlikely to be protective, it is not clear if these mycobacterial components elicit protective type T-cell responses. In animal models it has been shown that 65,000 MW heat-shock proteins do not impart protective immunity to challenge with virulent mycobacteria.[32]

Since the inoculum of live mycobacteria also contains some proportion of dead mycobacteria presumably in different stages of autolysis, it is possible that the lines generated by the dendritic cell-mediated approach would recognize a wide range of mycobacterial products. It would therefore be interesting to dissect out lines that recognize only live mycobacteria infected APC versus soluble mycobacterial proteins, as live mycobacteria are more efficient vaccines than killed ones.[33,34] T-cell clones that would respond to macrophages infected with replicating mycobacteria might be selected to study epitopes presented by infected APC. We are beginning such experiments using T cells from patients with tuberculosis.

ACKNOWLEDGMENTS

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Dendritic Cells Pulsed with Protein Antigens In Vitro Can Prime Antigen-specific, MHC-restricted T Cells In Situ

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Summary

T cells recognize peptides that are bound to MHC molecules on the surface of different types of antigen-presenting cells (APC). Antigen presentation most often is studied using T cells that have undergone priming in situ, or cell lines that have been chronically stimulated in vitro. The use of primed cells provides sufficient numbers of antigen-reactive lymphocytes for experimental study. A more complete understanding of immunogenicity, however, requires that one develop systems for studying the onset of a T cell response from unprimed lymphocytes, especially in situ. Here it is shown that mouse T cells can be reliably primed in situ using dendritic cells as APC. The dendritic cells were isolated from spleen, pulsed with protein antigens, and then administered to naive mice. Antigen-responsive T cells developed in the draining lymphoid tissue, and these T cells only recognized protein when presented on cells bearing the same MHC products as the original priming dendritic cells. In contrast, little or no priming was seen if antigen-pulsed spleen cells or peritoneal cells were injected. Since very small amounts of the foreign protein were visualized within endocytic vacuoles of antigen-pulsed dendritic cells, it is suggested that dendritic cells have a small but relevant vacuolar system for presenting antigens over a several day period in situ.

The immunologic activity of T lymphocytes is directed to antigens presented by MHC products on the surfaces of other cells termed APC (1-4). While many cell types are capable of generating MHC–peptide complexes and presenting these to primed T cells, it is evident that the dendritic cell subset of APC greatly accelerates the early sensitization phase of the immune response. This has been noted in vitro with transplantation (5-7) and viral (8, 9) antigens, and in situ using contact (10, 11) and transplantation antigens (12-14). Nevertheless, experimental studies of T cell sensitization in situ to antigens that require processing typically utilize artificial adjuvants rather than viable APC. Whenever bulk spleen cells have been used as APC in the absence of adjuvants, it has not been possible to restrict the sensitization to antigens in association with MHC products of the injected cells (15, 16). Therefore presentation in situ likely involves host rather than injected APC.

Kurt-Jones et al. used B cells as APC in situ to reverse a lack of T-cell responsiveness in mice that had been suppressed chronically with anti-μ antiserum (17). The B cells, when given simultaneously with antigen in CFA, appeared to be capable of priming MHC-restricted T cells in some but not all cases. In contrast, Lassila et al. (18) reported that in a chicken system B cells could not present antigens to T cells in situ.

Likewise, in primary antibody responses to hapten-carrier conjugates in vitro, dendritic cells and not B cells are required as APC early in the immune response (19).

Here we have assessed the capacity of antigen-pulsed dendritic cells to sensitize the T cells of an unprimed individual. We find that specific priming occurs, and that the sensitized T cells are restricted to recognize antigen on the MHC products of the presenting dendritic cells. These results suggest that dendritic cells are "nature's adjuvant." They are capable of delivering exogenous antigens, most likely as complexes of peptides on surface MHC products, directly to naive T cells in situ.

Materials and Methods

Mice. BALB/C × DBA/2 (CxD2)F$_1$ (H-2^d), C3H/HeJ (H-2^k), and (C3H × DBA/2)F$_1$ mice, 6–12 wk old and of both sexes, were purchased from The Trudeau Institute, Saranac Lake, NY.

Proteins. The antigens tested were spermwhale myoglobin, conalbumin, human gamma globulin, ovalbumin (Sigma Chemical Co., St. Louis, MO) and rhodamine-modified ovalbumin (Molecular Probes, Eugene, OR).

Culture Medium. For the preparation and antigen-pulsing of dendritic cells, the medium was RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 5% FCS, 50 μM 2-ME,

and 20 μg/ml gentamicin. For assessing T cell proliferative responses in vitro, the medium was Click's medium (Irvine Scientific, Santa Ana, CA) supplemented with 0.5% heat-inactivated mouse serum, 50 μM 2-ME, and 20 μg/ml gentamicin.

Antigen "Pulsing" of APC. As will be evident in Results, it was necessary to expose fresh rather than cultured dendritic cells to a foreign protein to successfully charge these APC with antigen. Adherent cells from a low buoyant density fraction of spleen were prepared (20–22) and cultured overnight (12–18 h) in medium to which 0.1 mg/ml of protein antigen was added. After overnight culture, the dendritic cells were purified by rosetting most of the contaminants (macrophages, B cells) with antibody-coated erythrocytes (21, 22). In experiments to be reported elsewhere (23), we have found that fresh dendritic cells purified on a FACS can be charged with antigen with just a 3-h exposure. However, the longer overnight "pulse" (12–18 h) was used, since it simplified the purification of the dendritic cells and improved their APC function. To ensure that the dendritic cells had been successfully pulsed with antigen, we did conventional restimulation assays using primed lymph node T cells (see Results). The other APC populations that were pulsed with foreign proteins were resident peritoneal cells, maintained in Teflon beakers to reduce macrophage adherence, and unfractionated spleen cells. In some experiments, 4 mg of protein was given to mice intravenously or intraperitoneally, spleen dendritic cells were isolated as described (24), and these in vivo pulsed APC were readministered to naive mice.

Priming with Antigen-pulsed APC In Situ. Antigen-pulsed APC were washed at least three times in RPMI 1640 and administered in PBS at a dose of 2–60 × 10⁵ cells in a volume of 25–40 μl into the fore or hind footpads. Generally, the antigen-pulsed APC were administered on one side, and the contralateral footpads served as the control. The control footpads were injected with APC that either had not been antigen-pulsed or were pulsed with a noncross-reacting protein (see Results). At varying times thereafter, but usually at day 5, the draining popliteal or brachial lymph nodes were removed, teased into a cell suspension, and challenged with antigen in vitro at 1–100 μg/ml. 3 × 10⁵ cells were cultured in triplicate in flat-bottomed microtest wells (No. 25860; Corning Glassworks, Corning, NY). DNA synthesis was measured on the 3rd day after exposure to [³H]TdR (specific activity, 6.0 Ci/mM) at 4 μCi/ml for 12–16 h. Unprimed lymph nodes never showed a response to the antigens we studied. The cells that responded by DNA synthesis in primed mice were shown to be primarily CD4⁺ Thy-1⁺ cells by treatment with appropriate mAb and complement before the assay for DNA synthesis.

MHC Restriction of In Vivo Primed T Cells. C3H × DBA/2 or A × DBA/2 F₁ (Iaᵏ × Iaᵈ) mice were primed with antigen-pulsed dendritic cells from either parental strain. 5 d later the draining lymph nodes were taken, and the cell suspensions were treated with mAb J11d anti-B cell and dendritic cell (22) and B21-2 anti-I-A plus rabbit complement (Pel-Freeze Biologicals, Rogers, AR) to deplete lymph node APC. The cells were then cultured at 3 × 10⁵ cells per microtest well in triplicate with graded doses of irradiated (1,000 rad ¹³⁷Cs) parental or F₁ spleen cells as APC with or without antigen. As will be evident in the Results, responsiveness was observed primarily when antigen was presented by the same parent that was used to prime the animals. To verify that the response was class II MHC-restricted, blocking studies with culture supernatants of anti-Ia mAbs were performed. The mAbs were B21-2 anti-Iaᵈ and 10-2.16 anti-Iaᵏ, both available at the American Type Culture Collection, Rockville, MD (TIB 229 and TIB 93, respectively).

Pinocytosis of Protein Antigens. Rhodamine-modified ovalbumin

proved to be a sensitive protein for visualizing pinocytosis by the weakly endocytic dendritic cells. Uptake was apparent after an overnight exposure to 0.1 mg/ml. Little or no uptake was evident at 0.02 mg/ml, or after a 2-h exposure, using fluorescence microscopy with a Zeiss Axiomat equipped for epifluorescence. The other tracers which we tested, which showed less and sometimes no uptake at the light microscopic level, were FITC-dextran, lucifer yellow, and horseradish peroxidase all at 0.1 mg/ml, the dose used to charge the APC with antigen. The positive control for active pinocytic activity was provided by resident macrophages in peritoneal washouts (see Results).

Results

Conditions for Pulsing Mouse Dendritic Cells with Protein Antigens In Vitro. We began with sperm whale myoglobulin, for which prior studies had defined an immunodominant region in the H-2ᵈ mouse corresponding to residues 106–118 (25, 26). Spleen adherent cells, which include dendritic cells, macrophages, and B cells, were cultured with or without native myoglobin overnight (16–24 h). The dendritic cells were then enriched by a standard method (21, 22) and tested for their capacity to stimulate myoglobin-primed T cells. As shown before (27), dendritic cells that had been cultured overnight without antigen were able to present peptide fragments, but presented native protein only weakly (Table 1, compare group 3 with groups 1 and 2). However, if the dendritic cells had been exposed to protein during the overnight culture, the antigen-pulsed APC vigorously stimulated the primed T cells [Table 1, group 4]. Similar findings were made with other proteins (human gamma globulin, conalbumin, and ovalbumin), but we did not have active peptide fragments for these antigens. Similar results also were obtained if the dendritic cells were cultured for 2 d before use (Table 1, compare group 7 with groups 5 and 6). However, it was noted that the antigen pulse was best if given during the first rather than the second day of culture (Table 1, compare groups 8–10). Once pulsed, the dendritic cell maintained immunogenicity for at least a day in culture (Table 1, group 10). We conclude that freshly isolated dendritic cells can be successfully pulsed with a variety of soluble protein antigens in vitro, but that it is important to administer the antigen shortly after isolating the dendritic cells from the spleen.

Antigen-pulsed Dendritic Cells Sensitize T Cells In Situ. After exposure to one of four different proteins, as above, the dendritic cells were injected into the left hind foot pad; companion unpulsed dendritic cells were injected into the right side. The draining popliteal lymph nodes from groups of three to five mice were taken 5 d later and tested for responses in vitro to each of three different proteins. To avoid responses to FCS components, which were present during the time that the dendritic cells were pulsed with antigen, the lymph node cells were cultured in the presence of mouse rather than fetal calf serum.

For each protein, the lymph node draining the site of antigen-pulsed, dendritic cell deposition developed specific antigen responsiveness (Table 2). If we injected two populations of dendritic cells, each pulsed with different proteins, then the lymph node cells acquired reactivity to both antigens.

Table 1. *Conditions for Pulsing Dendritic Cells with a Foreign Protein In Vitro*

Group	Culture of dendritic cells before use as APC*	Antigen[‡] during the APC-T coculture	DNA synthesis by antigen-primed T cells[§] to graded doses of dendritic cells[∥]			
			3×10^4	10^4	3×10^3	10^3
1	0–24 h, no antigen	None	3.9	1.9	0.8	0.2
2	0–24 h, no antigen	Myoglobin	7.6	1.6	0.5	0.2
3	0–24 h, no antigen	Myopeptide	45.9	20.2	6.9	1.2
4	0–24 h, myoglobin	None	91.6	37.9	10.8	4.2
5	0–48 h, no antigen	None	3.6	1.4	0.7	0.3
6	0–48 h, no antigen	Myoglobin	0.3	0.6	0.3	0.3
7	0–48 h, no antigen	Myopeptide	39.2	18.2	4.7	0.7
8	0–48 h, myoglobin	None	81.8	27.3	8.9	3.2
9	0–24 without myo, 24–48 with myo	None	15.0	4.4	1.7	0.7
10	0–24 with myo, 24–48 without myo	None	88.5	27.6	9.8	4.0

* Low density spleen adherent cells, which are a partially enriched population of dendritic cells (20–22), were cultured for 1 or 2 d in medium supplemented with antigen (100 µg/ml sperm whale myoglobin; Fluka) where indicated. After culture, contaminating macrophages and B cells were removed by rosetting with antibody-coated red cells.

‡ No antigen, myoglobin (5 µM), or myoglobin peptide 105–118 (2.5 µM), was added to the coculture of antigen-pulsed dendritic cells and myoglobin-primed T cells (below).

§ Antigen-primed T cells were prepared as follows. Mice were primed with 5 µm of myoglobin in CFA in the footpads. Brachial and/or popliteal lymph nodes were taken 5 d later and cell suspensions were prepared by teasing the nodes with forceps. The cell suspensions were cultured at 5 × 10⁶ cells/well in 24-well trays in 1.5 ml Click's medium supplemented with 0.5% mouse serum, 2 mM L-glutamine, 50 µM 2-ME, and 5 µM myoglobin. 10 d later the contents of the flask were applied to Ficoll (Sigma) columns to collect viable lymphoblasts. By using T cells that had been expanded in vitro, rather than fresh lymph node cells, we could obtain populations that responded strongly to re-stimulation with specific antigen on dendritic cells, without the syngeneic mixed leukocyte reaction that typically elevates background DNA synthesis when lymph node cells are cultured with unpulsed dendritic cells.

∥ 3 × 10⁴ myoglobin-primed T blasts (see §) were cultured with graded doses of dendritic cells (see *) [³H]TdR was added at 48–64 h to measure DNA synthesis. Data are mean cpm [³H]TdR uptake × 10⁻³ for triplicate wells; standard deviations were <10% of the mean. The experiment was repeated once with similar results.

In these latter experiments, there was some development of antigen reactivity in the nondraining lymph node (Table 2, last pair of antigens).

In experiments that are not shown, the antigen-reactive lymph node cells were CD4⁺ T cells primarily, since >80% of the reactivity could be eliminated with either anti-Thy-1 or anti-CD4 mAb and complement. The sensitizing capacity of dendritic cells was reproducible, in that individual mice each gave responses of comparable magnitude.

In kinetic studies, the draining lymph node cells became responsive over a 5-d period to the specific protein that had been used to pulse the injected dendritic cells (Fig. 1). Antigen specificity was maintained at all time points, i.e., if the left foot pad had been injected with dendritic cells pulsed with human gamma globulin, the left popliteal node developed specific responsiveness to human gamma globulin but not to other proteins (Fig. 1, *left*). Likewise, the right popliteal node developed responsiveness to the protein used to pulse the dendritic cells that were injected into the right food pad (Fig. 1, *right*).

When primed mice were rechallenged with antigen-pulsed dendritic cells but in a site distal to that used for the original priming (front vs. hind foot pad), the lymph node draining

this second site showed an accelerated or "memory" type response to the appropriately pulsed dendritic cells (Fig. 2). Responsiveness to antigen was apparent on the second day and virtually disappeared by the third.

Dendritic cells were also charged with a 2-h pulse of protein antigen in vivo as recently described (24). The dendritic cells were purified from the spleen using the FACS and injected into naive mice. Specific priming to the protein that originally had been given systemically to the dendritic cell donor was then observed (Table 3). We conclude that dendritic cells that had been pulsed with protein antigen in vitro or in vivo are capable of sensitizing CD4⁺ T cells from naive mice to that protein.

APC Requirements for Successful Priming In Situ. Dendritic cells were compared with two standard populations that have been used in many studies of antigen presentation in vitro. These were suspensions from spleen (a rich source of B lymphocytes) and peritoneal cavity (a rich source of macrophages and CD5⁺ B cells). The populations were pulsed with antigen for either 3 h or for 18 h in culture and administered in graded doses of 8 × 10⁵ to 5 × 10⁶ cells to the footpads of naive recipients (Table 4). Spleen cells were marginally effective, in that only the highest dose of 6 × 10⁶ cells induced

Table 2. *Dendritic Cells that Are Pulsed with a Protein Antigen In Vitro Specifically Prime Animals to that Protein In Situ*

Popliteal node	DC pulsed with:	No Ag	Conalbumin		Myoglobin		Hu × gamma globulin	
			100	10	100	10	100	10
					cpm × 10^{-3}			
Right	No Ag	0.4	0.7	0.4	0.7	0.6	3.2	2.0
Left	Conalbumin	0.3	126.1	92.7	1.3	0.8	3.4	0.9
Right	Myoglobin	0.2	0.4	0.2	32.3	33.9	1.4	0.9
Left	No Ag	0.2	0.5	0.3	0.3	0.4	1.9	0.1
Right	HGG	0.2	0.5	0.3	0.2	0.4	27.4	18.0
Left	No Ag	0.4	0.8	0.7	0.7	0.4	2.6	2.3
Right	Conalbumin + Myoglobin	0.5	58.7	18.5	40.0	42.7	3.7	2.4
Left	HGG	0.2	0.3	0.2	0.3	0.3	18.6	11.6
Right	Conalbumin	0.7	99.6	79.5	2.2	1.2	19.6	8.7
Left	Myoglobin + HGG	0.7	8.7	4.5	73.8	62.9	119.4	104.6
Right/left	No DC	0.1	0.2	0.2	0.2	0.2	0.6	0.3

Low density spleen adherent cells were cultured for 1 d with or without antigen, after which macrophages and B cells were depleted (21, 22). 2 × 10^5 dendritic cells were injected in the foot pads of groups of four mice. 5 d later, the draining popliteal nodes were taken and cell suspensions were prepared by teasing with fine forceps. 3 × 10^5 primed lymph node cells were cultured in flat-bottomed microtiter wells without antigen, or with the indicated antigens at 100 or 10 µg/ml. [^{3}H]TdR was added at 44–60 h to measure DNA synthesis.

Table 3. *Priming of Antigen Specific Lymph Node T Cells Using Dendritic Cells that Have Been Pulsed with Antigen In Vitro or In Vivo*

Dendritic cells [2 × 10^5] used to prime brachial nodes	No Ag	Conalbumin (µg/ml)			BSA	OVA
		100	10	1	100	100
		cpm × 10^{-3}				
In vitro pulse[‡]						
Myoglobin	0.1	0.1	—	—	0.9	—
Conalbumin	0.4	155.4	148.8	119.3	3.3	1.1
Conalbumin pulse + 25 µg soluble Ag in paw	0.4	161.8	145.3	124.1	4.8	0.3
In vivo[§]						
Conalbumin	0.3	68.7	45.5	22.1	1.5	1.0

* 5 d after priming with dendritic cells in the front footpad, brachial lymph node cells were prepared and restimulated in culture with the indicated antigen. DNA synthesis was measured on the third day.
‡ Low density spleen adherent cells were pulsed with 100 µg/ml of protein overnight. Dendritic cells were then purified by depleting FcR$^+$ cells (21, 22).
§ Mice were given 4 mg conalbumin i.v. 2 h later, the spleens were taken, and dendritic cells were isolated using the FACS and the N418 mAb to murine CD11c (24). N418 primarily reacts with dendritic cells in mouse spleen (23).

Left, primed with HGG–DC   Right, primed with conalb–DC

O No Ag
□ HGG
△ conalb
◇ Myo
O BSA

DNA synthesis (cpm ^{3}H–TdR x 10^{-3})

Days after priming with dendritic cells
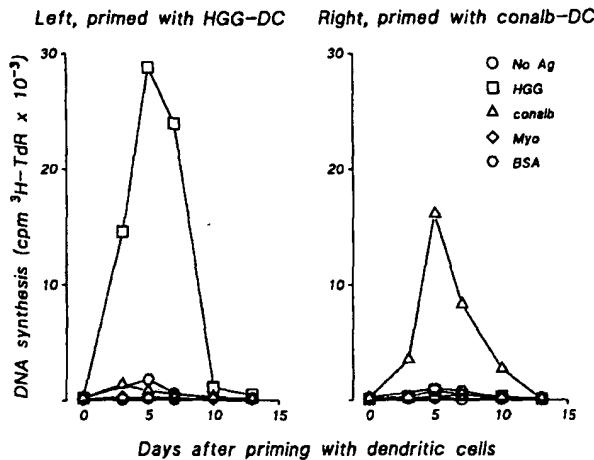
Figure 1. Kinetics of the primary response to antigen-pulsed dendritic cells. Low density spleen adherent cells were cultured with or without 100 μg/ml of the indicated proteins for 24 h. The cells were washed and 3 x 10^5, FcR$^-$ dendritic cells were injected into each foot pad. At the indicated time points, the draining popliteal and brachial lymph nodes were taken and cultured with the indicated proteins at 100 μg/ml in Click's medium supplemented with 0.5% mouse serum. DNA synthesis was measured on the third day. Lymph node cells that were primed with dendritic cells that had not been pulsed with antigen did not exhibit an antigen response (not shown, but see Table 2). The experiments were repeated twice with similar results.

a low level of responsiveness in the draining lymph node. Peritoneal cells were ineffective at all doses. Inocula of 2 and 5 x 10^5 dendritic cells had similar effects in situ, and the minimum dose capable of inducing some responsiveness was 3–8 x 10^4 dendritic cells (Table 4).

MHC Restriction of T Cells Primed by Antigen-pulsed Dendritic Cells. The finding that antigen-pulsed dendritic cells could prime naive animals to that antigen could be explained by a unique ability of dendritic cells to stimulate T cells directly in vivo, or alternatively, to transport antigens that were presented subsequently by host APC. The two possibilities could be distinguished by assessing whether the T cell sensitization process was restricted to antigens presented on the injected vs. host dendritic cells. We primed F$_1$ mice with antigen-pulsed dendritic cells from either parental strain and tested if the primed F$_1$ T cells could only be boosted with spleen APC from the original parent. It is known that most clones of T lymphocytes in an F$_1$ animal are restricted to antigens presented by one or the other parental MHC (28, 29). We used AxDBA/2 (H-2^a x H-2^d) or C3HxDBA/2 (H-2^k x H-2^d) F$_1$ recipients and primed with antigen-pulsed dendritic cells from each parental strain. 5 d later the F$_1$ lymph node cells were isolated, depleted of endogenous APC by treatment with anti-Ia and J11d mAb and complement, and challenged with APC from the F$_1$ or from either parent.

The F$_1$ T cells responded vigorously to antigen rechallenge in vitro with F$_1$ APC (Table 5). If parental strain APC were used, the rechallenge was far more effective with APC from the same parental strain that was used to sensitive the local node (Table 5). To show that the responses were re-

Days after injecting conalbumin–pulsed DC
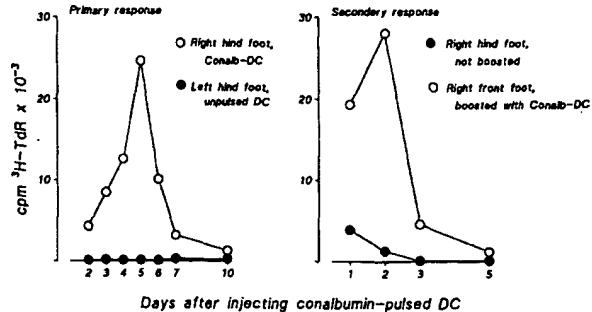
Figure 2. Secondary responses in mice that had been primed with antigen-pulsed dendritic cells. Conalbumin-pulsed dendritic cells (DC) were injected into the right hind foot pad as in Fig. 1, while the left hind foot pad received dendritic cells that were not pulsed with conalbumin. At the indicated time points (*left*), the draining lymph nodes were taken and boosted with protein antigen in vitro. Note that the primary response peaks at day 5 and subsides by day 7–9, as in Fig. 1. Companion groups of mice were then boosted on day 12 with antigen-pulsed DC, but in the right front foot pad to look for a secondary response (*right*). The experiment was repeated twice with similar results.

stricted to the class II MHC molecules of the sensitizing dendritic cells, we verified that a mAb to I-A^d blocked the restimulation of F$_1$ T cells primed with H-2^d APC but not with H-2^a APC, while mAb to I-A^k blocked presentation by H-2^k APC but not H-2^d APC (Table 6).

Visualization of Antigen Uptake in Dendritic Cells. Since one pathway for antigen presentation likely involves endocytosis of the foreign protein followed by proteolysis and formation of peptide-MHC complexes (4), we monitored the extent to which dendritic cells could accumulate a protein that we could visualize, rhodamine-modified ovalbumin. When dendritic cells were pulsed overnight in 0.1 mg/ml of protein, it was evident that each cell had a small number of fluorescent granules, usually close to the nucleus (Fig. 3, *left*). Macrophages in contrast were much more heavily labeled after exposure to rhodamine-ovalbumin (Fig. 3, *right*). These results suggest that the strong APC function of dendritic cells in situ is associated with the accumulation of only small amounts of the foreign protein.

To verify that the rhodamine tracer that was being visualized in the above experiments was in fact relevant to immunogenicity in situ, we charged dendritic cells with rhodamine-modified ovalbumin and administered the cells to mice. 5 d later cells from the draining lymph node were tested for antigen responsiveness. Interestingly, responsiveness developed in a dose-dependent manner (Fig. 4, *left*) but it was specific for the conjugate rather than the free ovalbumin carrier (Fig. 4, *right*). Therefore, the rhodamine group that we were monitoring in the uptake studies above (Fig. 3) was relevant to the antigen-specific sensitization that was occurring in situ.

Discussion

Several features of dendritic cells help explain the sensitizing function of these APC in situ (30). One is their capacity to

Table 4. *Dendritic Cells Are Specialized to Present Antigens In Vivo*

	Myoglobin-pulsed APC used to prime in vivo		Proliferation of primed lymph node cells with			
				Myoglobin		Conalbumin 100 μg/ml
Exp.	Cell type	Number of cells	No Antigen	5 μm	0.5 μm	
1	Spleen cells	5 × 10⁶	0.1	5.6	0.8	0.2
		2 × 10⁶	0.1	2.1	0.2	0.2
		8 × 10⁵	0.1	0.1	0.1	0.2
	Dendritic cells	5 × 10⁵	0.1	32.5	28.1	0.3
		2 × 10⁵	0.1	29.9	17.6	0.1
		8 × 10⁴	0.1	4.2	0.6	0.1
	Peritonal cells	2 × 10⁶	0.1	0.1	—	0.1
		8 × 10⁵	0.1	0.1	0.1	0.1
	None	—	0.1	0.1	0.1	0.1

	Conalbumin-pulsed APC used to prime in vivo		Proliferation of primed lymph node cells with				
				Conalbumin (μg/ml)			Ovalbumin 100
	Cell type	Number of cells	No Antigen	100	10	1	
2	Spleen cells	2 × 10⁶	0.3	9.8	6.3	2.0	0.2
		1 × 10⁶	0.3	2.1	0.5	0.2	0.2
		5 × 10⁵	0.3	0.3	0.3	0.2	0.2
		2.5 × 10⁵	0.3	0.3	0.2	0.2	0.2
	Dendritic cells	5 × 10⁵	0.3	56.3	54.2	19.6	0.3
		2.5 × 10⁵	0.3	47.2	40.1	12.1	0.3
	Peritonal cells	2 × 10⁶	0.2	0.4	0.3	0.3	0.2
		1 × 10⁶	0.2	0.3	0.3	0.2	0.2
		5 × 10⁵	0.2	0.3	0.2	0.2	0.2
		2.5 × 10⁵	0.2	0.2	0.2	0.2	0.2

Three different populations of APC were exposed to 100 μg/ml of myoglobin for 16 h (Exp. 1) or conalbumin (Exp. 2), washed, and administered into the footpads of naive mice at the indicated doses. 5 d later the draining lymph nodes were taken, and the cell suspensions were restimulated with antigens as shown. Data are cpm [³H]TdR uptake × 10⁻³ at 48-60 h. Data are not shown for APC that were *not* pulsed with antigen before administration to mice, since the proliferative responses were all 0.3 or less.

capture and retain antigens, a feature that seems to be short-lived in culture (Table 1) (27). Another is their capacity to form stable conjugates with resting, antigen-specific T cells and induce the development of functional T lymphoblasts (31-33). A third feature is the ability to home via the lymph and the blood (34-36) to the T-dependent areas of lymphoid organs. These functions together should allow the antigen-pulsed dendritic cell to select clones of specific T cells from the recirculating pool (37).

The number of dendritic cells that were effective in our experiments, 1-3 × 10⁵, is not large when other variables are considered. The efficiency with which these cells leave the injection site and home to the draining lymph node may be very small, since Kupiec-Weglinski et al. (34) reported that only 1-2% of a dose of ¹¹¹In-labeled dendritic cells were retained within the draining lymph node. This means that a few thousand dendritic cells may carry out the sensitization of T cells reported here. A similar conclusion was reached in studies of pancreatic islet transplantation. There, a maximum of 2,000-4,000 dendritic cells seemed responsible for sensitizing mice across an MHC barrier (13). The efficiency of these APCs likely relates to the fact that they home to

Table 5. *Primed F_1 T Cells Are Restricted to the Parental Strain of the Dendritic Cells Used in Priming*

Exp.	Spleen APC used to boost F_1 T cells *in vitro* Strain	Dose	Primed with Ia^k DC − Ag	Primed with Ia^k DC + Ag	Primed with Ia^d DC − Ag	Primed with Ia^d DC + Ag
				cpm × 10^{-3}		
A: A × DBA/2 F_1 T cells	A × DBA/2 ·[Ia^{kxd}]	3 × 10⁵	0.6	11.4	0.3	11.8
	A [Ia^k]	3 × 10⁵	0.5	22.4	0.1	0.1
		5 × 10⁵	0.8	29.6	0.3	0.6
	BALB/C × DBA/2 [Ia^{dxd}]	3 × 10⁵	0.3	2.7	0.2	22.9
		5 × 10⁵	0.4	3.6	0.6	65.4
B: C3H × DBA/2 F_1 T cells	C3H × DBA/2 [Ia^{kxd}]	1.25 × 10⁵	0.2	14.7	0.3	58.2
		2.50 × 10⁵	0.6	21.8	0.5	73.0
		5 × 10⁵	1.1	25.0	3.4	67.1
	BALB/C × DBA/2 [Ia^{dxd}]	1.25 × 10⁵	0.2	0.5	0.2	46.7
		2.50 × 10⁵	0.8	1.3	0.9	76.7
		5 × 10⁵	2.0	2.2	2.5	69.0
	C3H [Ia^k]	1.25 × 10⁵	0.1	13.3	0.4	0.8
		2.50 × 10⁵	0.4	31.6	1.2	1.4
		5 × 10⁵	1.8	26.2	2.9	3.4

F_1 mice (Ia^{kxd}) were primed with 3 × 10⁵ dendritic cells (DC) that had been pulsed with 100 μg/ml of conalbumin. The dendritic cells were from either parental strain. 5 d later, the lymph nodes were taken and cell suspensions were prepared by teasing with fine forceps. These suspensions were treated with B21-2 anti-Ia, and J11d mAb and complement to deplete endogenous APC, and then plated at 3 × 10⁵ cells in flat-bottomed microtiter wells in Click's medium with 0.5% mouse serum. Irradiated spleen cells (1,500 rad ¹³⁷Cs) from either parental strain, or the F_1 strain, were then added at the indicated doses as a source of APC. Conalbumin was (+ Ag) or was not (− Ag) added to the cultures at 100 μg/ml. DNA synthesis was measured at 44–60 h.

Table 6. *Priming by Antigen-pulsed Dendritic Cells In Vivo is MHC-restricted*

Strain of dendritic cells used to prime C3H × D2 F_1 T cells*	Strain of spleen used to present antigen in vitro‡	DNA synthesis in presence of:§ no Ab	αCD4	αIa^k	αIa^d
			cpm × 10^{-3}		
C3H, H-2^k	C3H, H-2^k	55.9	8.5	11.7	42.6
	C3H, H-2^k	12.5	1.1	3.2	10.1
BALB/C × DBA/2, H-2^d	BALB/C × DBA/2, H-2^d	155.5	12.0	128.0	23.8
	C3H × DBA/2 (H-2^{kxd})	86.2	9.0	41.7	12.9

* 2 × 10⁵ dendritic cells, pulsed with conalbumin, were injected into C3H × DBA/2 F_1 [H-2^k × H-2^d] mice. 5 d later, lymph nodes were treated with antibodies to Ia and J11d + complement to deplete endogenous APC. 3 × 10⁵ T cells were then cultured per well. The experiment was repeated once with similar results.
‡ 3 × 10⁵ spleen cells from the indicated strains were irradiated (¹³⁷Cs, 1,000 rad) and added to the primed T cells.
§ DNA synthesis was measured by adding [³H]TdR at 66–72 h. Conalbumin was added in all the experimental cultures that are shown, since proliferation in the absence of antigen was <1 × 10³ cpm. The mAbs tested for blocking activity were GK1.5 αCD4, 10-12.16 αI-A^k, and B21-2 αI-A^d.

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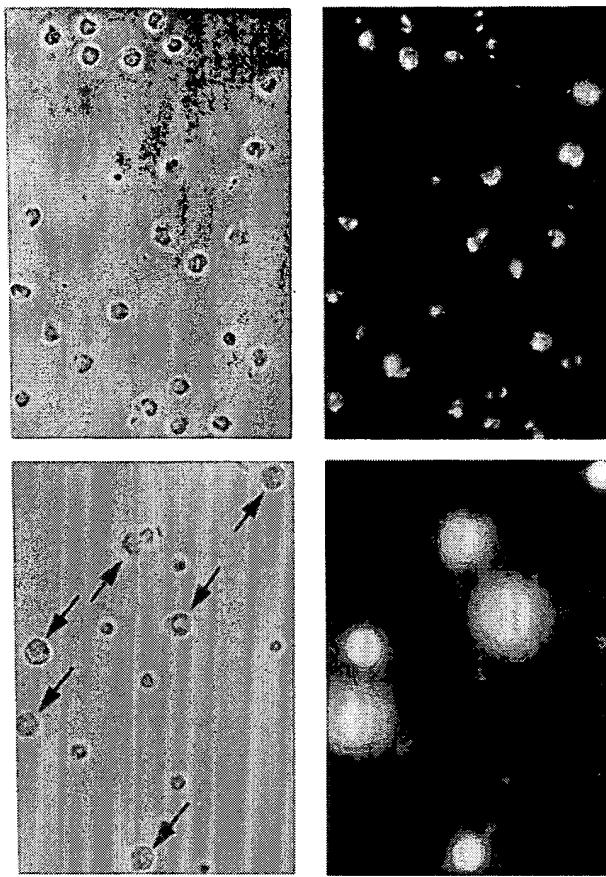
Figure 3. Detection of endocytic activity by antigen-pulsed dendritic cells. Low density spleen adherent cells were cultured overnight in 0.1 mg/ml of rhodamine-modified ovalbumin. The FcR⁻ dendritic cells were enriched and attached to glass slides coated with poly-L-lysine (21). The dendritic cells (*top*) were uniform in cytologic appearance (*left*) and each contained small numbers of rhodamine-labeled granules. In parallel, peritoneal cells were also exposed RITC-OVA. The large macrophages (*bottom, arrows*), as verified by strong indirect immunofluorescent staining with mAb to the M1/70 CD11b antigen, were intensely labeled with the endocytic tracer (*right*). ×300.

an optimal site in the lymphoid tissue and are each capable of binding and activating large numbers of T cells even with relatively small amounts of ligand on the dendritic cell surface (38).

Since the endocytic apparatus may provide an important route for the processing and presentation of exogenous antigens (4), it is of interest that the poorly endocytic dendritic cell (Fig. 3) is nonetheless extremely active in antigen presentation. This suggests to us that the endocytic apparatus of dendritic cells may be specialized to present antigens, whereas in macrophages, the bulk of the endocytic activity results in antigen clearance and degradation (39). In the experiments described here, it is formally possible that non-dendritic cells in the adherent spleen preparation were processing and regurgitating peptides onto dendritic cell MHC molecules, but in a recent study (24), we presented evidence that such a phenomenon is undetectable in our cultures. In addition, we have also prepared highly enriched populations of dendritic cells fresh from spleen, using the FACS, and found them to be potent APC for priming T cells in situ (23).

Once CD4⁺ or CD8⁺ T cells pass a control point that involves activation by dendritic cells, the sensitized T cells efficiently interact with other types of APC to carry out various effector functions that are critical for T cell–mediated immunity. For example, CD4⁺ T blasts that are induced by dendritic cells can interact in an antigen-specific way to make B lymphocytes grow and respond to B cell stimulating factors (40), and to make macrophages synthesize IL-1 (41, 42). CD8⁺ T blasts that are induced by dendritic cells can kill other APC as targets (43, 44). These "effector" aspects of the immune response may be restricted to inflammatory sites in situ, given the evidence that sensitized T cells that are produced in lymphoid tissues emerge into the lymph (45) and can move via the blood stream to inflammatory sites (46, 47). It is of interest that dendritic cells are not known to have any effector or antigen elimination functions, in contrast to other APC, such as B cells and macrophages which can re-
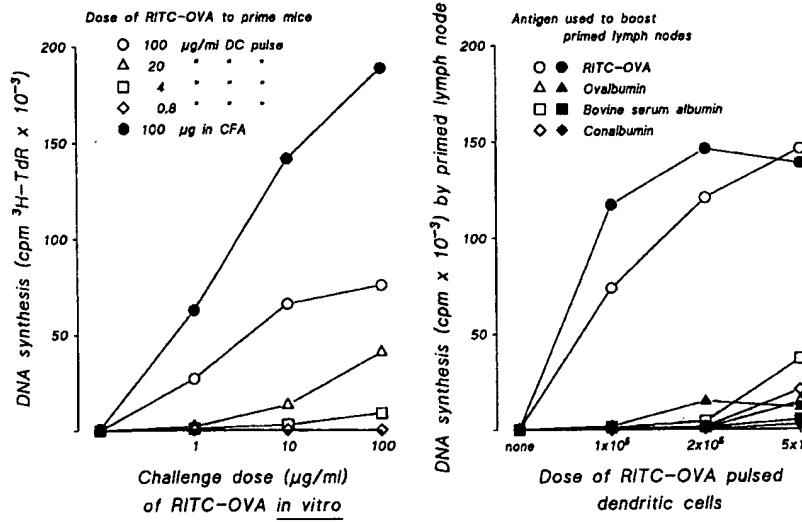
Figure 4. Rhodamine-ovalbumin (*RITC-OVA*)-pulsed dendritic cells prime mice in situ. Low density spleen adherent cells were exposed to graded doses of RITC-OVA (*left*) or to 0.1 mg/ml RITC-OVA (*right*) for 16 h, and then the dendritic cells were purified and injected at a dose of 3 × 10⁵ into fore and hind foot pads (*left*) or in graded doses (*right*) into groups of three mice. 5 d later the draining lymph nodes were taken and boosted with graded doses of RITC-OVA (*left*) or RITC-OVA vs. other proteins (*right*). Only the data with 100 μg/ml protein are shown on the right, with the popliteal and brachial nodes in open and closed symbols, respectively. [³H]TdR uptake was measured at 48–60 h.

lease antibody or kill microorganisms. The specialized role of dendritic cells seems to be to sensitize T lymphoblasts, which then interact with other APC.

Extracorporeal pulsing of antigens onto dendritic cells may provide a new approach to immunization *in situ*, a goal that previously could be approached only empirically with adjuvants. By using dendritic cells as a natural adjuvant, one has an opportunity of having the APC select those epitopes on a complex antigen that can be presented by a given individual's MHC products. This strategy provides a physiologic selection of immunogen that may serve as an important alternative to the empirical search for immunogenic peptides that is being pursued so actively in recent years.

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The Sensitization Phase of T-Cell-Mediated Immunity[a]

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Major emphasis in research on T-cell-mediated immunity has been placed on the
related topics of antigen presentation and T-cell recognition of antigen plus MHC. As
summarized in several other reports presented herein (Grey, Gefter), antigen presenta-
tion may entail several steps. "Nominal" antigens such as soluble proteins and
infectious agents are processed to form peptides 8–18 amino acids in length. The
peptides then associate with polymorphic MHC molecules. The secondary and tertiary
structures of MHC molecules form a single peptide-binding site that includes most of
the polymorphic residues, the latter restricting the type of peptide that can be bound.
Finally, the peptide-MHC complex on the presenting cell surface is recognized by the
alpha-beta heterodimeric receptor on the T lymphocyte.

Many aspects of antigen presentation and recognition are best studied with
monoclonal populations of lymphocytes such as T-cell clones and T-T hybrids. These
monoclonal populations are maintained in culture for long periods and do not
necessarily correspond to resting lymphocytes. A good example is that many T-cell
clones respond vigorously to exogenous growth factors, whereas resting T cells do not.
Therefore, the study of antigen presentation to T-cell clones and hybrids does not
necessarily provide a complete picture of the requirements for immune responses by
resting T cells.

We were attracted to this question because of findings that have emerged from
several laboratories on the function of dendritic cells as antigen presenting cells. (See
reference 1 for a review.) In tissue culture, the addition of small numbers of dendritic
cells to purified lymphocytes—usually in a ratio of 1:30 to 1:100— leads to active
mixed leukocyte reactions, antibody production to foreign red cells and hapten-carrier
conjugates, proliferation to mitogens, and proliferative and cytotoxic responses to
hapten-modified cells. In situ, antigens in association with dendritic cells induce graft
rejection, contact sensitivity, and antibody formation. In several of these responses,
other antigen presenting cells such as macrophages and B lymphocytes have little or no
activity.

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At this time, the only known function of dendritic cells is to *sensitize* T cells, that is, to convert small inactive lymphocytes into large active lymphoblasts. The lymphoblasts release lymphokines and/or kill targets. T blasts interact with other presenting cells such as B lymphocytes and macrophages, leading to effector functions such as antibody formation and microbial killing, which are required for antigen elimination. In contrast to these other antigen presenting cells, dendritic cells are not known to have effector functions. For example, they typically do not phagocytose or clear immune complexes, produce antibodies, or secrete interleukin-1 (IL-1).

In this report, we address recent findings on the mechanisms whereby dendritic cells are specialized to act as accessory cells early, or in the *sensitization* phase, of cell-mediated immunity. Sensitization clearly requires antigen presentation and recognition, but it likely requires other events. Dendritic cell function will be discussed in terms of: the cell's high levels of surface MHC products; molecules required for the formation of dendritic–T-cell contacts; release of lymphocyte-activating factors such as IL-1; and cytokines that may help mobilize active dendritic cells from nonlymphoid organs.

EXPRESSION OF MHC PRODUCTS AND ANTIGEN PRESENTATION

Most studies of dendritic cell function have used populations from lymphoid tissues, blood, and afferent lymph. These "lymphoid" dendritic cells uniformly express high levels of class I and II products of the MHC. In the past, expression of MHC products was monitored by quantitative binding studies with radioiodinated monoclonal antibodies (mAb), autoradiography, and immunofluorescence. It is now feasible to stain and study small numbers of cells by flow cytometry, which provides a rapid and quantitative view of the dendritic cell surface (FIG. 1). This figure illustrates the very high levels of class I (here H-2K; 100 times background) and class II (I-A/E; 600 times background) MHC products on mouse spleen dendritic cells (FIG. 1, left column) and also that dendritic cells react weakly, if at all, to mAb that detect either macrophage- or lymphocyte-restricted epitopes (F4/80, B220, thy-1, CD4; middle column, FIG. 1). The one dendritic cell-specific reagent, 33D1, stains dendritic cells weakly but is cytolytic in the presence of rabbit complement.[2] When a panel of mAb to specific receptors is studied, the 2.4G2 Fc receptor antigen is not detectable, but low levels of other receptors (C3bi and IL-2 receptors, LFA-1; FIG. 1, right) are noted. The surface phenotype of dendritic cells differs markedly from that of macrophages, which typically show lower levels of MHC products and a different constellation of differentiation antigens and receptors. Notably, macrophages from peritoneal cavity (FIG. 2) and spleen (not shown) have relatively high levels of 2.4G2 (Fc receptor) and Mac-1 or M1/70 (C3bi receptor), but little or no 7D4-3C7 antigen (55-kd, low affinity IL-2 receptor).

It is important to point out that the enrichment methods used to prepare dendritic cells do not themselves select for a high content of MHC products. Instead, the procedures select for cells with the unusual shape and surface activity (formation and movement of cell processes) of these leukocytes. These dendritic cells have low levels of Fc receptors and lack many important macrophage and lymphocyte markers, features that are used to develop a variety of purification techniques for dendritic cells in specific tissues. In every case, the enriched populations express high levels of class I and II MHC products. For example, in mouse spleen and thymus (in preparation), dendritic cells are most readily enriched from populations that initially are firmly adherent. After overnight culture, dendritic cells are separated from adherent macrophages by selecting cells that lack abundant Fc receptors and lose the adherence

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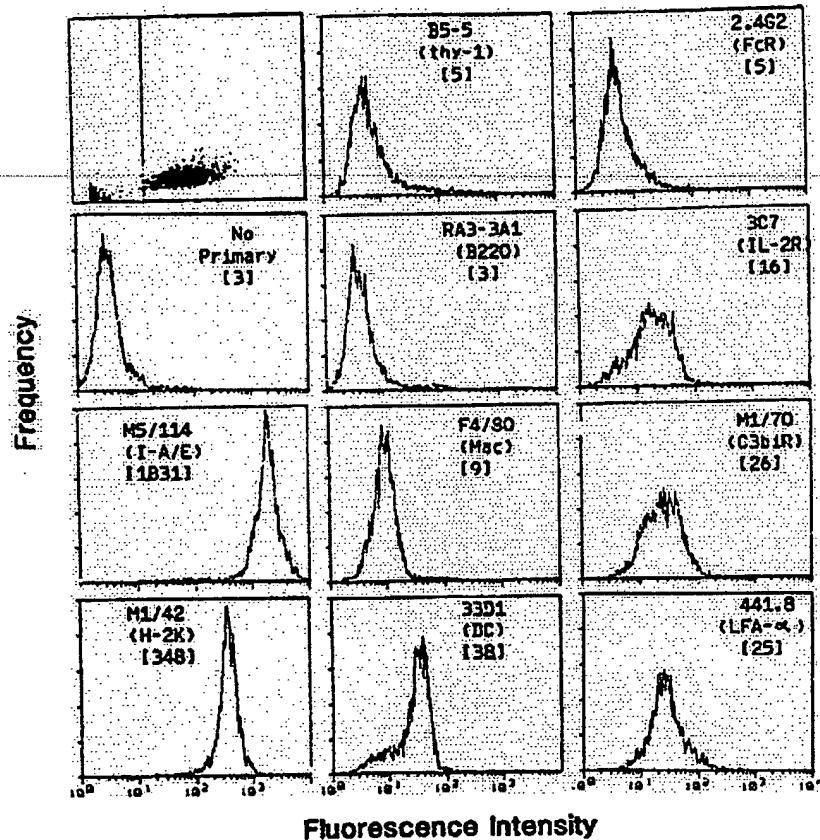
FIGURE 1.

FIGURES 1 and 2. Flow cytometric analysis of the cell surface antigens of mouse spleen dendritic cells (FIG. 1) and peritoneal macrophages (FIG. 2). The dendritic cells were Fc receptor negative, spleen-adherent cells,[3] whereas the macrophages were resident cells from the peritoneal cavity. (Similar results are observed with macrophages purified by plastic adherence and/or maintained for a day in culture; not shown.) Side versus forward light scatter dot plots for each population are shown in the *top left panels*. Note that spleen dendritic cells have the light scattering properties of lymphocytes, whereas the macrophages are selected by gating for cells with much larger forward and side scatter. (Most cells with the macrophage-restricted F4/80 and M1/70 antigens fall to the right of the horizontal gate.) Each population was exposed to hybridoma culture supernatants followed by fluorescein-conjugated mouse anti-rat Ig (Boehringer). The monoclonal antibodies[47] (clone name, antigen recognized, median fluorescence) are given on each frequency versus fluorescence tracing. The data and antibodies are arranged in three groups: antibodies to MHC products are on the *left;* antibodies to cell-specific antigens in the *middle;* and antibodies to defined receptors on the *right*. Not shown are several antibodies that did not stain either macrophages or dendritic cells. These included reagents[47] to Lyt-1, Lyt-2, L3T4, interdigitating cell antigen, and KJ/16 T cell receptor antigen.
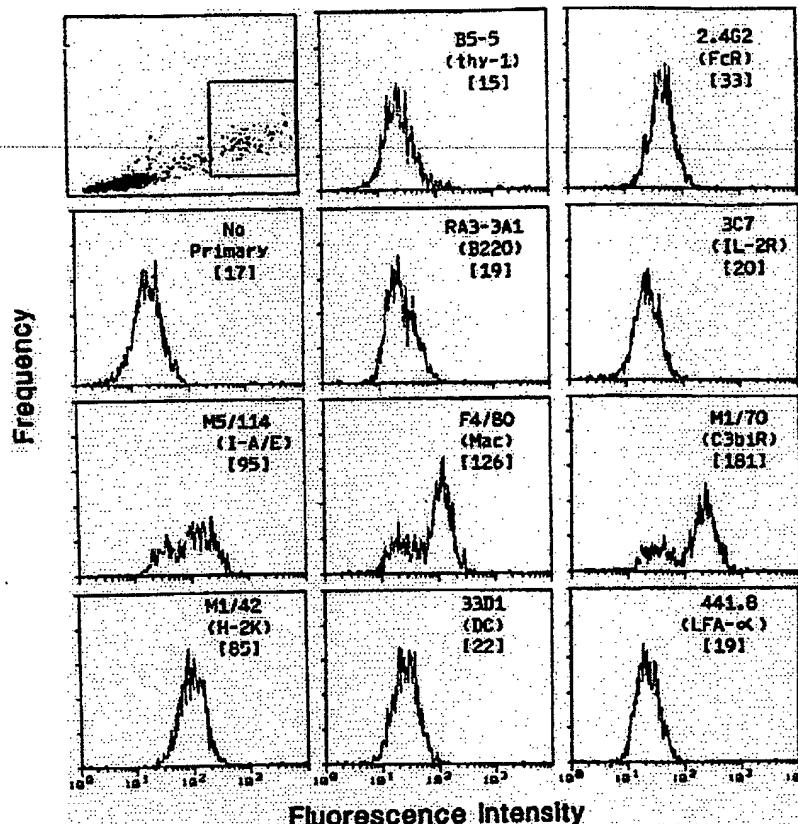
FIGURE 2.

property. The nonadherent Fc⁻ cells are almost all dendritic in shape and exhibit the phenotype and high levels of MHC products outlined in FIGURE 1.[3,4] Dendritic cells in suspension from rat lymph or rat lymphoid organs do not adhere to glass or plastic even temporarily. If one depletes lymphocytes, one again enriches for a dendritic population that is rich in transplantation antigen.[5,6] Human blood dendritic cells occupy an intermediate status in terms of adherence. To achieve the best yields, it is best to start the enrichment procedure after 1–2 days in culture when most dendritic cells are nonadherent.[7]

Not only are the levels of MHC products on dendritic cells very high, but also these levels seem to be under distinct controls. This was noted some years ago when it was discovered that MHC products could be upregulated on macrophages, but not on dendritic cells, by T-cell-derived lymphokines.[8] This finding has been verified with recombinant gamma-interferon and quantitative binding studies with ¹²⁵I anti-Ia antibody (TABLE 1).

There are now instances in which immature forms of dendritic cells have been found such as in bone marrow,[9] epidermis,[10,11] and the nonadherent fraction of thymus

suspensions.[12] The term "immature" is used, because these populations must be cultured for 1–2 days before optimal levels of surface Ia and accessory function are expressed. In thymus and epidermis, maturation is driven by factors such as IL-1 and GM-CSF.[12,13]

Although lymphoid dendritic cells express high levels of MHC products, more work is needed on their ability to present "nominal" rather than "transplantation" antigens. The processing of various proteins and infectious agents may occur in endocytic vacuoles for class II-restricted antigens and perhaps along the exocytic pathway for class I-restricted antigens such as virus constituents. Lymphoid dendritic cells are not actively endocytic when challenged with a number of soluble tracers and particulates, and have yet to be shown to be permissive for any virus infection. Therefore, how do dendritic cells perform an intracellular processing function to present antigens in association with MHC products? One possibility that we are considering is that most dendritic cells that are isolated from lymph and lymphoid organs have already processed antigens in nonlymphoid tissues *in situ* and have migrated to the lymphoid organs with peptides to be presented to appropriate clones of antigen-specific T cells. In other words, antigen processing and presentation by

TABLE 1. Recombinant Immune Interferon Enhances Expression of Ia Antigens on Splenic Macrophages But Not Dendritic Cells[a]

Spleen Cells	Interferon	Binding Sites/Cells $\times 10^{-5}$	
		Experiment 1	Experiment 2
Macrophages	−	85	58
	+	180	175
Dendritic cells	−	224	214
	+	231	150

[a]Low density spleen-adherent cells were cultured for 2 days ± 10 U/ml of recombinant murine gamma-interferon. The cells were then separated into firmly adherent macrophages and nonadherent dendritic cells as described.[6] Each population was exposed to radioiodinated B21-2 mAb to Ia antigens under saturating conditions on ice as described.[8]

dendritic cells may be regulated and may be confined to those intervals in which dendritic cells are exposed to antigens in inflammatory sites.

BINDING TO ANTIGEN-SPECIFIC T LYMPHOCYTES

One of the challenging unknowns in cell-mediated immunity is: how does a receptor in the plasma membrane of one cell, the T lymphocyte, come in contact with antigens displayed on the surface of another cell, the antigen presenting cell? Dendritic cells clearly have solved this challenge. During antibody formation to T-dependent antigens and during the mixed leukocyte reaction, the bulk of the response is carried out in clusters of dendritic cells and lymphocytes.[14-19] Typically the clusters are isolated on day 1 or 2 of the immune response. The response then develops from the aggregates over the next 2–3 days. Clustering with dendritic cells is a feature of both primary and recall ("memory") phases of responses in culture. Similar clusters have been identified *in situ* in delayed type hypersensitivity[20] and allograft rejection.[21]

Several features of the clustering phenomenon have been amenable to study *in vitro*. Clustering is efficient with respect to both the dendritic cell and the antigen-specific T cell. The movement of dendritic cells into clusters is best visualized using

fluorescent carbocyanine dyes as a vital label.[19,22] The movement of antigen-specific lymphocytes is monitored by indirect criteria. The response of isolated clusters is observed to be comparable to that of mixtures of clusters and nonclusters, implying that most of the responsive cells are in the aggregates. In a mixed leukocyte reaction, rechallenge experiments performed on nonclustered T cells will demonstrate a depletion of specific or first party reactivity but responsiveness to a donor displaying different or third party MHC products.[15,16]

In primary responses by either CD4+ or CD8+ subsets, T-cell clustering seems specific for dendritic cells. Clusters do not form with macrophages or B lymphocytes,[15,16,23] and there is as yet no direct evidence that resting T cells can establish contacts with these presenting cells in an antigen-specific fashion.

A different situation pertains when lymphoblasts rather than resting T cells are studied. Enriched populations of antigen-specific T lymphoblasts are released from dendritic-T-cell clusters, and these lymphoblasts can bind[23] and activate other antigen presenting cells. In fact, a good criterion for the sensitization process is that the T cell acquires the capacity to bind to other targets so that the efferent limb of the immune response can be carried out. An example is the antibody response to hapten-carrier conjugates.[24] If T cells are primed with dendritic cells and carrier protein in vitro, the T blasts apparently activate B cells in a direct, antigen-specific, and MHC-restricted manner. This kind of information supports the view that there are two stages in cell-mediated immunity. During the first or afferent-sensitization stage, contacts are established with dendritic cells, and T-cell growth and function begin. In the second or efferent-effector stage, the T lymphoblast binds and influences the appropriate target to provide effector function. We just exemplified the latter in the context of antibody formation by B cells, but similar findings pertain to the induction of macrophage IL-1 by sensitized T lymphoblasts (Koide; Bhardwaj; in preparation).

The molecular mechanism of dendritic-T-cell clustering is not clear. Both LFA-1 (CD11a) and CD4 are thought to be important accessory molecules for the binding of lymphocytes to presenting cells. However, antibodies to LFA-1 and CD4 do not seem to block the initial binding of dendritic cells to resting or activated T lymphocytes.[25] These antibodies block the subsequent function of the dendritic-T-cell conjugate. Anti-LFA-1 can disassemble the conjugate and make it very susceptible to shear forces, whereas anti-CD4 blocks lymphoblast formation and lymphokine release.

To summarize, we have a situation in which antigen alone does not seem to be the first "signal" for the formation of cell-cell contacts early in the immune response, because antigen on macrophages and B cells does not initiate clustering. Antigen must be available on these presenting cells, given their capacity to interact with T lymphoblasts in a specific way.[15,23] LFA-1 and CD4 also are involved in the communication between presenting cells and lymphocytes, but these molecules do not appear to be the first signals. We are working on the hypothesis that there are novel molecules that mediate an early reversible interaction between dendritic cells and T cells, and that this interaction is followed by antigen recognition and by the function of CD4 and LFA-1.[25] To identify clustering molecules, we are screening antidendritic cell hybridomas for the capacity to block clustering with T cells. This strategy has yet to prove successful. Immunization with dendritic cells primarily leads to the development of anti-MHC antibodies rather than dendritic cell-specific reagents.

RELEASE OF LYMPHOCYTE-ACTIVATING FACTORS

Once dendritic-T-cell contacts have developed, what comes next? Do dendritic cells release a factor that is required for the lymphocyte response, or is the entire

process regulated by the interaction between cell surface components? The evidence is now extensive that lymphoid dendritic cells do not produce one specific activating factor, IL-1. Dendritic cells from mouse spleen, human blood, and the exudates of joint effusions of rheumatoid arthritis have been studied.[26,27] In each case, IL-1 production is not detected, whereas mononuclear phagocytes actively make this cytokine. A useful approach involves immunolabeling with a rabbit antiserum to a human IL-1 beta peptide. The antibody stains individual cells with cytoplasmic IL-1[28] and is as sensitive as the D10 bioassay in detecting IL-1 in human monocytes.[27] However, no IL-1 can be detected in dendritic cells when clustered with responding lymphocytes.

Nonetheless, IL-1 amplifies T-dependent proliferative responses in culture as long as the cultures contain dendritic cells. It seems that one of the principal effects of IL-1 is exerted on the dendritic cell.[22] Dendritic cells can be pulsed for 8–18 hours with IL-1, washed, and then added to the mitogenesis assays. In both peripheral and thymocyte proliferative responses, pulsing dendritic cells before use as accessory cells is as effective as adding IL-1 continuously to the cultures.[12,22] The pulsed dendritic cells are not blocked with a neutralizing anti-IL-1 antibody, but the antibody does block the action of IL-1 during the pulsing step.[12]

These results raise two new points with respect to dendritic cell function. The first is that the secretory capacity of dendritic cells and macrophages is different. More products need to be studied. It is not known if dendritic cells make other important effector cytokines that are made by macrophages such as cachectin-tumor necrosis factor and IL-6. A second point is that dendritic cell function can be amplified by cytokines. Interestingly this enhanced function is not associated with an increase in expression of Ia. Instead, the amplifying effect of IL-1 seems to be due to the more efficient clustering of dendritic cells and T lymphocytes before the onset of mitogenesis.[22]

MOBILIZATION, MOVEMENT, AND MATURATION OF TISSUE DENDRITIC CELLS

Antigens are typically deposited in nonlymphoid organs. Where does the T cell learn that antigen is present and begin to respond? Is antigen presented in the periphery and there is an influx of T cells into the inflammatory site, or must antigen move centrally to the lymphoid organ via the afferent lymph to select antigen-specific clones in the T-dependent areas? The latter would seem to be a predominant site for T-cell sensitization for three reasons (see reference 1 for a review): blastogenesis and the production of sensitized T cells are detected early in the draining lymphoid organs; sensitization for contact sensitivity and skin allografts is blocked if the afferent lymphatics are severed; and the continual recirculation of T cells through the T area provides a favorable site for the selection of specific clones by antigen.

If dendritic cells are required for T-cell sensitization, are dendritic cells present at the site of antigen deposition? Because of a lack of mAb that can be used to stain dendritic cells in section, there is a lack of detailed evidence on tissue distribution. However, a combination of criteria—cytologic features by electron microscopy, expression of abundant class II molecules, and absence of certain cytochemical and antigenic markers of macrophages and lymphocytes—indicates that dendritic cells are present in the interstitial tissues of heart,[29,30] lung,[31] and urinary tract[29,32] as well as the T-dependent regions of lymphoid organs.[33,34]

A second feature relates to the migratory properties of dendritic cells. Balfour et al.[35-37] found that dendritic or veiled cells are present in rabbit and pig afferent lymph. Other studies indentified dendritic cells in lymphatics draining contact sensitivity sites

in guinea pig[38] and mouse[39,40] and in afferent lymphatics from rat intestine.[641] Austyn and colleagues[42,43] labeled mouse spleen dendritic cells and followed their migration *in situ*. Dendritic cells were injected into the bloodstream or foot pads of syngeneic recipients, and migration was monitored using an indium label for quantitation and a DNA-binding fluorochrome for histologic localization. The dendritic cells homed to the T-dependent areas. In spleen, dendritic cells accumulated in the marginal zone by 3 hours and in the T area by 24 hours. Labeled dendritic cells also were applied to frozen sections and noted to bind selectively to the marginal zone region at the periphery of the white pulp.[43]

To assess the functional properties of tissue dendritic cells more directly, Schuler *et al.*[44] undertook a study of mouse epidermal Langerhans cells in culture. Langerhans cells are the principal, if not the only, Ia$^+$ cell in the epidermis[44] and are responsible for the observed accessory function of epidermal suspensions. (See references 45 and 46 for a review.) Schuler *et al.*[44] found that Langerhans cells had many of the properties of dendritic cells, but only after 1–3 days of co-culture with keratinocytes.[10,11] The cultured Langerhans cells were nonadherent and nonphagocytic, and had low levels of Fc receptors. During culture, the levels of class I and II MHC products increased fivefold,[47] and the accessory function for primary T-dependent responses increased 10- to 30-fold.[10,11]

The development of accessory function in cultured Langerhans cells seems entirely dependent on exogenous cytokines.[13,48] The required cytokines are present in the conditioned medium from cultured keratinocytes and from stimulated macrophages and T cells. The active keratinocyte product has proven to be granulocyte-macrophage colony stimulating factor (GM-CSF). Anti-GM-CSF neutralizes the activity of keratinocyte-conditioned medium, and recombinant GM-CSF is the only purified cytokine that mediates Langerhans cell maturation *in vitro* with half-maximal activity at 0.5 pmol. Once the Langerhans cell has developed in response to GM-CSF, accessory function cannot be blocked by anti-GM-CSF.[13] Heufler *et al.*[48] found that IL-1 enhances the effect of GM-CSF by about twofold, but IL-1 by itself does not influence Langerhans cell viability and function. Several other factors, including macrophage- or M-CSF and IL-3, have no effect.

The observations summarized in this section suggest that an important element in the sensitization phases of the immune response is the influence of cytokines such as GM-CSF on the properties of tissue dendritic cells. GM-CSF is produced by many cell types, not just sensitized T cells, so that it may be released as a very early event in inflammatory sites where antigens are deposited.

SUMMARY

Many cell-mediated immune responses appear to develop in two phases: A *sensitization phase* in which unprimed or memory T cells interact with dendritic cells to become active lymphoblasts, and an *effector phase* in which the T lymphoblasts and other presenting cells interact to eliminate the antigen. Antigen presentation is essential to both phases. Here we review several features that are pertinent to the special sensitization role of dendritic cells. First, dendritic cells from lymphoid tissues, blood, and lymph (lymphoid dendritic cells) express very high levels of class I and II MHC products, and these levels cannot be increased by exposure to cytokines such as immune interferon. Second, dendritic cells efficiently cluster antigen-specific T cells during primary responses. Other presenting cells, like macrophages and B lymphocytes, do not form clusters but do bind to sensitized T lymphoblasts. Dendritic-T-cell binding is not inhibited by mAb to CD4 and LFA-1 antigens. It is suggested that a

dendritic-cell-specific molecule is required. Third, it is not yet clear if dendritic cells make a "lymphocyte activating factor." However, IL-1 is not produced, even when dendritic cells are in contact with responding T cells. Fourth, dendritic cells have the capacity to migrate from the tissues and move to T-dependent areas. Epidermal Langerhans cells represent a reservoir of tissue dendritic cells but seem to be immunologically immature. The viability and accessory function of the Langerhans cell greatly depend on a single cytokine, granulocyte-macrophage colony stimulating factor (GM-CSF), leading to the proposal that GM-CSF is critical in mobilizing active dendritic cells at the onset of a cell-mediated immune response.

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Granulocyte-Macrophage Colony-stimulating Factor Promotes Differentiation and Survival of Human Peripheral Blood Dendritic Cells In Vitro

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Abstract

Interest in human dendritic cells (DC) has been heightened recently by the discovery that this cell type is a primary target of the human immunodeficiency virus, the causative agent of AIDS. DC are bone marrow–derived cells with an extraordinarily potent ability to promote the immunological activity of T lymphocytes. Unfortunately, since DC constitute < 0.5% of peripheral blood mononuclear cells and die within a few days of their isolation, they are not readily accessible to study. We report here that granulocyte-macrophage colony-stimulating factor (GM-CSF), a cytokine with well-recognized effects on granulocyte and macrophage maturation, profoundly affects the morphology and viability of DC isolated from peripheral blood. GM-CSF not only promotes DC survival but also induces DC differentiation to mobile, reversibly adherent cells with long-branched projections. DC cultured in GM-CSF survive for up to 6 wk and retain their ability to stimulate the proliferation of T cells in allogeneic and autologous mixed leukocyte reactions. (*J. Clin. Invest.* 1990. 85:955–961.) autologous mixed leukocyte reaction • cytokine • Langerhans cells • macrophage • T lymphocyte

Introduction

Bone marrow–derived dendritic cells (DC)[1] are highly efficient at presenting foreign antigen to T cells, thus initiating an immune response. Such cells are distinguishable from other mononuclear cells on the basis of their morphology and surface phenotype (1–4). In peripheral blood DC appear as large (> 15 μm) irregularly shaped cells, but in tissues such as skin and lymphoid organs DC have multiple dendrite-like cellular projections. Although no DC-specific surface antigens have been described, DC in both blood and tissues can be readily distinguished from other leukocytes on the basis of their dense expression of class II major histocompatibility complex deter-

minants and their lack of the monocyte/macrophage-associated antigen Leu M3, the T cell antigen CD3, natural killer cell antigen CD16, and B cell antigens, CD20 and CD22. Interest in DC has grown in recent years partly as a result of studies in mice suggesting that this cell type, in contrast to macrophages, can prime naive T cells to become antigen-specific T helper cells or virus-specific cytotoxic cells (5–7). Moreover, recent studies in humans have demonstrated that DC can be infected with the human immunodeficiency virus (HIV), the causative agent of AIDS, and may serve as a major reservoir for the virus, in vivo (8, 9). Unfortunately, detailed studies of the functions of human DC and the effects of HIV infection on this cell type have been constrained by the fact that DC constitute < 0.5% of peripheral blood mononuclear cells and survive for only a few days in vitro.

In the current report, we show that peripheral blood DC differentiate into multibranched cells and survive, functionally intact, for prolonged periods in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF), a cytokine known primarily for its effects on the maturation of granulocyte and macrophage precursors (10, 11). The approach used here should make it possible to undertake a broad range of studies of DC heretofore not possible.

Methods

Isolation of peripheral blood DC, monocytes, T cells, and B cells. Human peripheral blood DC were obtained with a previously described procedure (4) with the modifications indicated below. Briefly, mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation (12). Low-density and high-density mononuclear cells were separated in a four-step discontinuous Percoll (Pharmacia LKB, Uppsala, Sweden) gradient. 75% (7 ml), 50.5% (16 ml), 40% (4 ml), and 30% (3 ml) dilutions of stock isoosmotic solution of Percoll (1.130 g/ml) in Dulbecco's calcium- and magnesium-free PBS containing 5% heat-inactivated pooled human serum (DPBS/HS) were layered sequentially. 100–200 × 10⁶ mononuclear cells suspended in the Dulbecco's PBS/ human serum were overlayered onto the gradient and centrifuged at 1000 × g for 25 min at 4°C. Low-density cells, mostly monocytes, were collected from the interface over 50.5% Percoll solution (density 1.065 g/ml), while high-density cells were collected from the interface between 75% and 50.5% Percoll solutions. T cells forming spontaneous rosettes with sheep red blood cells were removed from the high-density cell fraction by Ficoll-Hypaque centrifugation (13). The remaining high-density non–T cells (including DC) were suspended in Teflon vessels in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, and 10% heat-inactivated pooled human serum (hereafter designated complete medium [CM]) and cultured at 37° in 10% CO₂ in air for 2 d. Thereafter, the cells were centrifuged over a second discontinuous Percoll gradient consisting of 75% (2 ml), 50.5% (4.5 ml), 40% (1 ml), and 30% (1 ml), and the DC were contained entirely within the low-density fraction.

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1. *Abbreviations used in this paper:* CM, complete medium; DC, dendritic cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; MLR, mixed leukocyte reaction; Tsn, T cell supernatant.

This fraction was further depleted of contaminating macrophages by a solid-phase absorption (panning) procedure on human IgG-coated Petri dishes (4). By cytofluorographic analysis using two-color staining with phycoerythrin and fluorescein-conjugated monoclonal antibodies, 50–70% of the recovered cells stained brightly with a monoclonal anti–HLA-DR antibody (CA141), and did not stain simultaneously with any of the following antibodies defining leukocyte subsets: Leu M3 (monocyte/macrophages), OKT3 (anti–CD3, T cells), Leu 5 (anti–CD2, T and natural killer cells), Leu 12 (anti–CD19, B cells), Leu 16 (anti–CD20, B cells), and Leu 11c (anti–CD16, natural killer cells). A final panning procedure (14) simultaneously utilizing Leu M3, OKT3, Leu 12, and Leu 11c antibodies yielded > 70% of cells with the above staining characteristics. Final yields of DC were 0.08–0.6% of starting mononuclear cells.

Other cell populations were obtained during the same procedure and further purified as follows: The monocyte enriched "primary" low-density fraction was refloated on Percoll and purified by panning on human IgG-coated Petri dishes. The "secondary" high-density B cell-enriched fraction was further purified by removing residual monocytes, T cells, and NK cells on Petri dishes coated with Leu M3, OKT3, and Leu 11c antibodies. The T cell-enriched fraction was depleted of residual monocytes by overnight adherence in plastic vessels at 37°C. During the 2-d isolation procedure, unfractionated peripheral blood mononuclear cells were maintained at 37°C in Teflon vessels.

By cytofluorographic analysis > 90% of the macrophage-enriched population stained with Leu M3 antibody, > 96% stained with OKM1, whereas ≤ 2% were CD3+ and ≤ 1% CD20+. 96% of this population stained with an anti–HLA-DR antibody (CA141), but the mean fluorescence was 1 log lower than that found in the DC-enriched fraction. The B cell fraction contained > 60% CD20+ cells, ≤ 1% CD3+, ≤ 3% CD16+ cells, and ≤ 1% Leu M3+ cells. > 95% of the cells in the T lymphocyte-enriched fraction were CD3+, while ≤ 1% were either CD20+ or Leu M3+.

T cell supernatant (Tsn). Tsn was obtained from cultures of T cells pulsed 18 h with 5 μg/ml PHA, washed four times, and maintained at 37°C for an additional 4 d.

Mixed leukocyte reactions. (MLRs). 20 × 10³, 10 × 10³, or 5 × 10³ DC, monocytes, or B cells were cultured for 0 or 18 d in microwells containing 0.2 ml CM alone or CM supplemented with 100 U/ml GM-CSF. Thereafter, the microtiter plates containing these cells were irradiated (3,000 rads from a cesium-137 source) and 50 × 10³ cryopreserved allogeneic or autologous T cells were added in CM alone or CM supplemented with 50 U/ml GM-CSF (0 d MLRs only). During the pre-MLR 18-d culture, each cell population was fed every 8 d with CM with or without GM-CSF. After 18 d, half the medium was removed and 50 × 10³ T cells in 0.1 ml CM were added. All MLRs were carried out at 37°C in 10% CO₂ in air. On the 6th day of culture 1 μCi per well [³H]thymidine (specific activity 6.7 Ci/mmol) was added to the wells, and 16 h later the cells were harvested with a MASH apparatus (Otto Hiller Co., Madison, WI) for determination of [³H]thymidine incorporation. Results represent the mean counts per minute±SD of five replicate cultures.

Results and Discussion

Using a modification of a multistep procedure initially described by Young and Steinman (4), we obtained highly enriched DC from human peripheral blood. The DC in these preparations are large (> 15 μm) and distinguishable by flow cytometry from other mononuclear cells on the basis of their high surface density of HLA-DR and absence of detectable T, B, or monocyte markers (Fig. 1). When dispersed in culture these cells rapidly (2–4 h) form aggregates, remain viable for a few days in clumps, and then die. When DC isolated in this manner were exposed to supernatants (Tsn) of T cells which
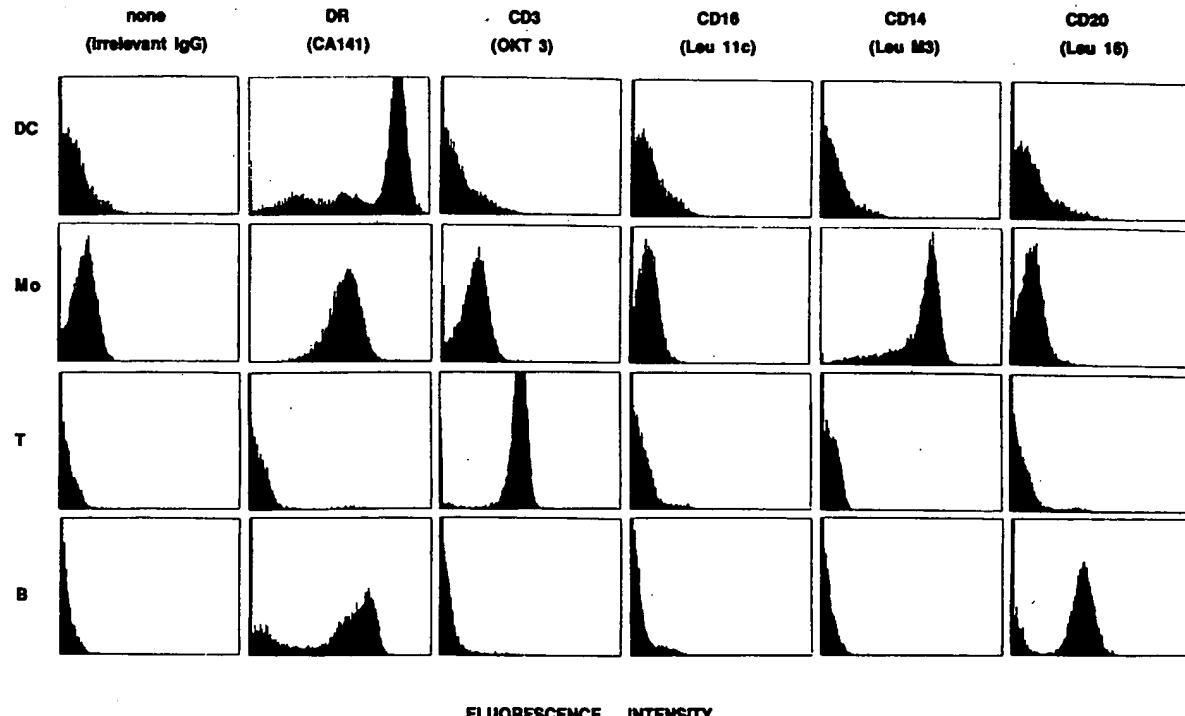
Figure 1. Cell surface markers on freshly isolated dendritic cells (DC), macrophages (Mo), and T and B lymphocytes (T and B) obtained from peripheral blood. Immunofluorescence analysis was performed on an Epics Profile II (Coulter Cytometry, Epics Division, Hialeah, FL). Histograms display number of cells versus intensity of fluorescence on a logarithmic scale. Each histogram represents an analysis of 10,000 cells.
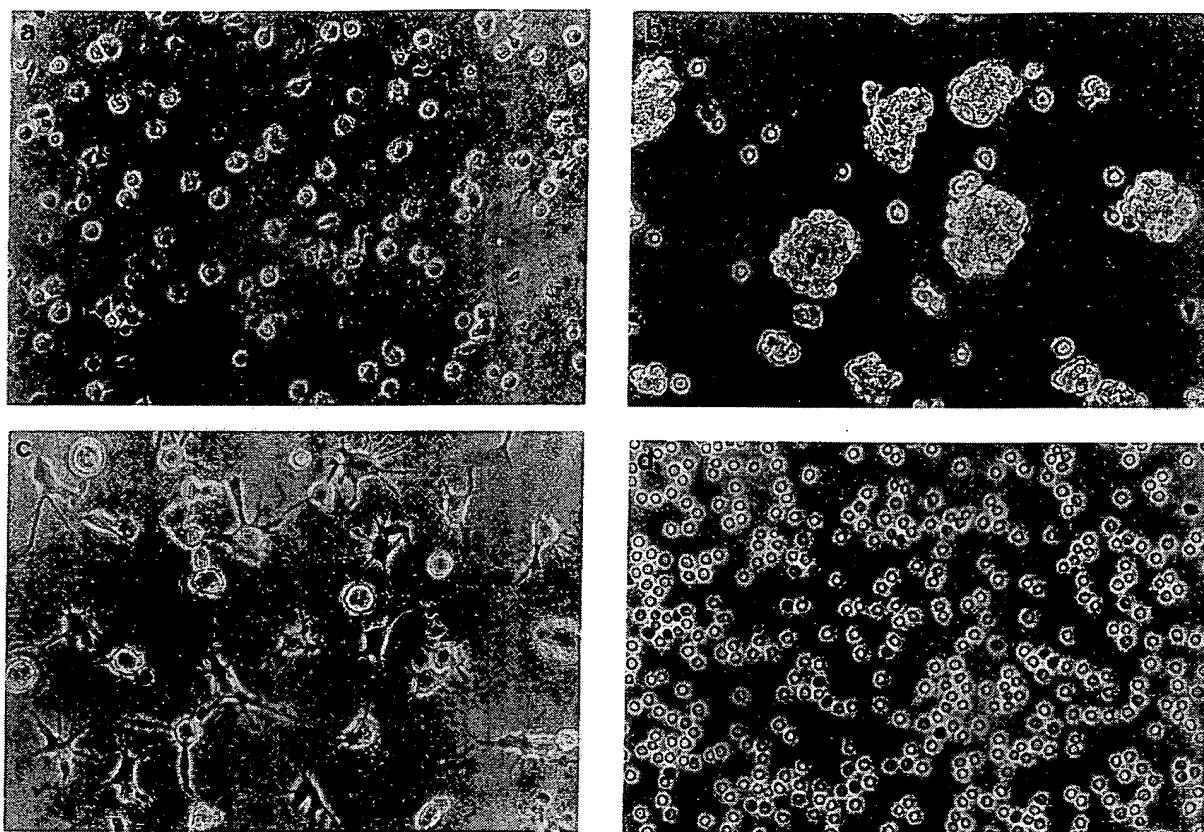
Figure 2. Morphology of human peripheral blood DC. DC were isolated from peripheral blood as described in Methods. Samples shown represent (*a*) fresh DC suspended in serum-free medium and centrifuged immediately to avoid aggregate formation, (*b*) DC aggregates formed within 2 h of culture in CM; (*c*) DC after culture for 21 d in CM supplemented with 100 U/ml native GM-CSF, and (*d*) fresh T cells (shown for comparison). For long-term culture, DC were suspended in CM supplemented with GM-CSF in 12-well dishes (Costar Data Packaging Corp., Cambridge, MA) at a final cell concentration of 0.15 × 10⁶/ml. Cultures were fed every 8 d with fresh CM containing GM-CSF. DC maintained in Tsn were identical in appearance to those maintained in GM-CSF. All photographs were taken through an inverted phase-contrast microscope (×143).

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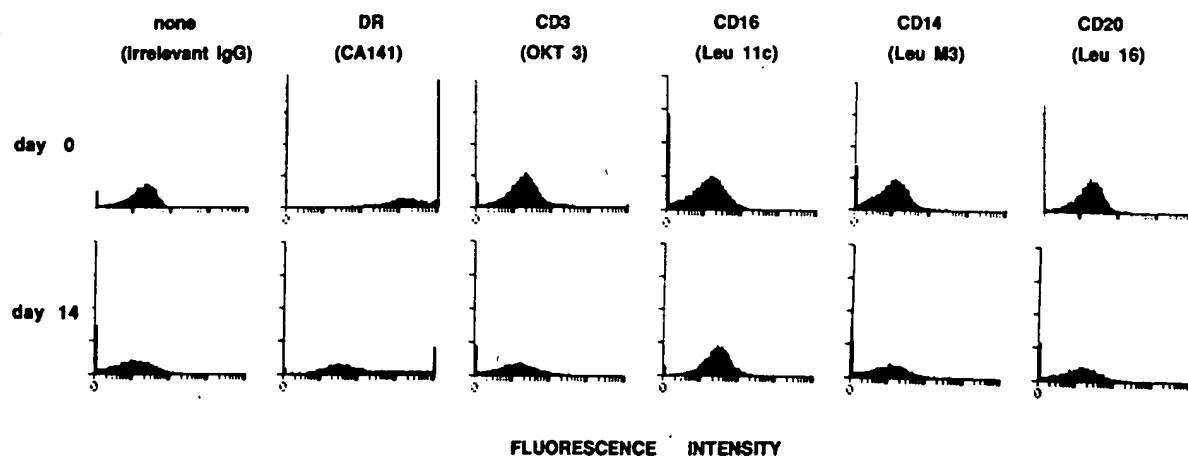
Figure 3. Comparison of cell surface markers on freshly isolated DC and DC cultured for 14 d in CM supplemented with 100 U/ml GM-CSF. Immunofluorescence was performed on a cell sorter (model 50 H, Ortho Diagnostic Systems, Inc., Raritan, NJ). Histograms display cell number versus fluorescence intensity on a logarithmic scale. Each histogram represents an analysis of ~ 5,000 cells.

had been stimulated with phytohemagglutinin (PHA), the clumped cells disaggregated, differentiated into reversibly adherent cells with long-branched processes (as in Fig. 2), and remained viable for > 1 mo in culture. The surface phenotype of DC maintained in Tsn for 2 wk was similar to that of freshly isolated DC, with widely variable staining for HLA-DR and no staining for other leukocyte markers (Fig. 3). Supernatants prepared as above from the T cells of 10 consecutive subjects had identical activity.

This result suggested that one or more T cell–derived cytokines induce DC differentiation and survival in culture. When a spectrum of purified human cytokines was tested for effects on DC, GM-CSF alone recapitulated the activity of Tsn (Table I). Purified native or recombinant GM-CSF promoted DC differentiation and prolonged survival in a dose-dependent

manner. Furthermore, by using a panel of neutralizing anti-cytokine antibodies, only those antibodies to GM-CSF blocked the effect of Tsn on DC (Table II). Complete blockade of Tsn activity was obtained with 50 NU/ml IgG anti-GM-CSF.

Peripheral blood DC cultured for 3–7 d with GM-CSF displayed an array of cell processes (Fig. 2) which tended to protrude and retract repeatedly over time. The adherence of DC to plastic induced by GM-CSF was reversible, inasmuch as the cells frequently detached from the surface of the culture vessel and readhered in a different location. As shown in Fig. 4, the number of differentiated (branched) DC increased as the concentration of GM-CSF in the culture increased. At any given concentration of the cytokine, however, the total number of viable cells as well as the number of branched cells per

Table I. Effect of Various Cytokines on DC Morphology and Viability in Culture

Factor	Source	Concentration in DC culture	Morphologic change	Duration of DC viability
				wk
Tsn	T cells + PHA for 5 d	1/2 vol/vol	+++	≥4
rIL-1α+rIL-1β	Immunex (Seattle, WA)	50 U/ml each	−	≤1
		20 U/ml each	−	≤2
		2 U/ml each	−	≤1
rIL-2	Cetus (Emeryville, CA)	10 U/ml	+/− or −	≤1
		2 U/ml	+/− or −	≤1
rIL-3	Genzyme (Boston, MA)	250 U/ml	−	≤1
		50 U/ml	−	≤1
		10 U/ml	−	≤1
rIL-4	Genzyme	50 U/ml	−	≤1
		20 U/ml	−	≤1
IL-6 (natural)	Endogen (Boston, MA)	100 U/ml	−	≤1
		50 U/ml	−	≤1
rIFNγ	Collaborative Research (Bedford, MA)	20–1,000 U/ml	−	≤1
rM-CSF	Cetus, ICN (Cleveland, OH)	5,000 U/ml	−	≤1
		1,000 U/ml	−	≤1
rG-CSF	Amgen (Thousand Oaks, CA)	50–1,000 U/ml	−	≤1
PDGF (natural)	Cellular Products (Buffalo, NY)	16 U/ml	−	≤1
Recombinant insulin-like Growth Factor	Collaborative Research	25 ng/ml	−	≤1
Transforming growth factor-β (natural)	Collaborative Research	2–20 ng/ml	−	≤1
GM-CSF (natural)	Endogen	500 U/ml	++++	5–6
		250 U/ml	++++	5–6
		100 U/ml	+++	5–6
		50 U/ml	+++	5
		20 U/ml	+	4
		10 U/ml	+	4
		2.5 U/ml	+/−	3
rGM-CSF (yeast)	Genzyme	250 U/ml	++++	5–6
		125 U/ml	+++	5–6

DC were cultured in CM at a concentration of 25,000 cells per well in flat-bottom 96-well tissue culture plates (Costar) in the presence of the indicated agent. DC morphology was determined by phase-contrast microscopy. The number of branching DC per well was scored as follows: −, no differentiated DC; +/−, occasional differentiated DC; +, number of branching DC approximately one-third that seen in wells with Tsn; ++, number of branching DC approximately two-thirds that seen in wells with Tsn; +++, number of branching DC approximately equal to that seen in wells with Tsn; ++++, number of branching DC greater than that seen in cultures with Tsn. The results shown represent a summary of 19 separate experiments.

Table II. Effect of Neutralizing Anti-Cytokine Antibodies on Morphologic Change of CD Induced by Tsn

Experiment	Reagent preincubated	Antibody used in preincubation	Antibody concentration	Antibody source	DC differentiation
1–3	Tsn	No antibody	—	—	+++
	Tsn	Preimmune IgG control	25 μg/ml	Endogen	+++
	Tsn	Anti–TNFα	250 NU/ml	Endogen	+++
	Tsn	Anti–IFNα	1650 NU/ml	ICN	+++
	Tsn	Anti–IFNβ	60 NU/ml	Lee Biomolecular Research Laboratories (San Diego, CA)	+++
	Tsn	Anti–IFNγ	250 NU/ml	Endogen	+++
	Tsn	Anti–IL-1	200 NU/ml	Endogen	+++
	Tsn	Anti–IL-2	86 NU/ml	Collaborative Research	+++
	Tsn	Anti–lymphotoxin	250 NU/ml	Endogen	+++
	Tsn	Anti–GM-CSF	250 NU/ml	Endogen	−
	Tsn	Anti–IL-6	250 NU/ml	Endogen	+++
4, 5	Tsn	No antibody	—		+++
	Tsn	Preimmune IgG control	40 μg/ml	Endogen	+++
4, 5	Tsn	Anti–GM-CSF	400 NU/ml	Endogen	−
	Tsn	Anti–GM-CSF	200 NU/ml	Endogen	−
	Tsn	Anti–GM-CSF	100 NU/ml	Endogen	−
	Tsn	Anti–GM-CSF	50 NU/ml	Endogen	−
	Tsn	Anti–GM-CSF	25 NU/ml	Endogen	+
	Tsn	Anti–GM-CSF	12.5 NU/ml	Endogen	++
	GM-CSF (200 U/ml)	Anti–GM-CSF	200 NU/ml	Endogen	−
	GM-CSF (200 U/ml)	Anti–GM-CSF	50 NU/ml	Endogen	+++

DC differentiation was scored as described in the footnote to Table I. Tsn or GM-CSF was preincubated for 5 h (experiments 1–3) or 2 h (experiments 4 and 5) with rabbit polyclonal IgG neutralizing antibody, and was then passed through a 0.2-μm filter. The concentration of immune-IgG during preincubation is shown in neutralizing units (NU) as provided by manufacturers. DC cultures in CM were supplemented with a half volume of Tsn or GM-CSF solution previously incubated with antibodies. TNF, tumor necrosis factor.

well remained stable over time, suggesting that GM-CSF does not cause DC to divide and proliferate. The possibility that some DC die and are replaced by DC developing from precursor cells present in the initial DC preparation cannot be ruled


Figure 4. Effect of GM-CSF concentration on number of differentiated (branched) DC. DC were cultured in 96-well flat-bottom plates (Costar) at a density of 20,000 cells per well, and viable, branched DC were identified and counted microscopically. The range of the total number of viable cells per well was 10,000–15,000 throughout the culture period.

out. Indeed, some nonadhering cells were able to adhere and display processes when transferred to another culture vessel (data not shown). Branched DC typically comprised 10–40% of the total number of viable cells in cultures supplemented with GM-CSF. The remaining cells consisted primarily of unbranched DC (DR+, Leu M3− cells capable of differentiating into branched adherent cells when transferred), and smaller numbers of macrophages and lymphoid cells.

DC differentiation to branching cells was not dependent on the multistep isolation procedure used for initial enrichment of DC from peripheral blood. If GM-CSF was added directly to cultures of unfractionated peripheral blood mononuclear cells, scattered cells with branching processes regularly appeared within a few days (not shown). Differentiated DC similar to those induced by GM-CSF were occasionally observed in cultures to which recombinant (r)IL-2 had been added. An abundance of rapidly proliferating lymphocytes was also observed in such cultures, attributable to an effect of IL-2 on T cells contaminating the initial DC preparation. We interpret this result as indicating that the effect of IL-2 on DC is most likely an indirect one, mediated by activated T cells producing GM-CSF. However, a direct effect of IL-2 on DC is not excluded.

No other cytokine or combination of cytokines tested induced DC differentiation. However, DC remained viable for up to 1 wk in cultures supplemented with IL-1, IL-3, or IL-4, and if GM-CSF or Tsn was added to such cultures, the surviv-
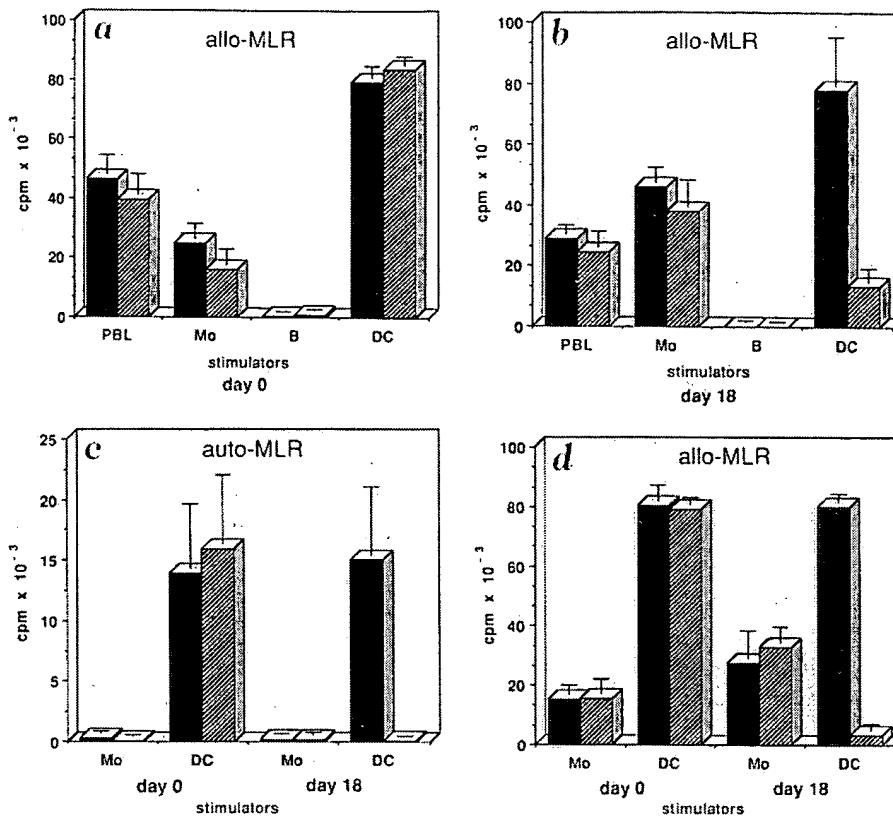
Figure 5. DC cultured in GM-CSF maintain their immunostimulatory function in MLR. MLRs were carried out between 5×10^4 T cell responders and fresh stimulator populations (day 0) or between T responders and stimulator cells which had been cultured for 18 d with GM-CSF (*solid bars*) or without GM-CSF (*hatched bars*). Day 0 MLRs were also performed in the presence (*solid bars*) or absence (*hatched bars*) of GM-CSF. (*a*) Fresh peripheral blood mononuclear cells (*PBL*), monocytes (*Mo*), B cells (*B*), and dendritic cells (*DC*) were cultured with allogeneic T cells. (*b*) 10×10^3 cells of each population were cultured for 18 d before being used as allogeneic stimulator cells. In a separate experiment, Mo and DC freshly isolated or cultured for 18 d were used to stimulate (*c*) autologous T cells or (*d*) allogeneic T cells. In this experiment 20×10^3 autologous and 5×10^3 allogeneic stimulators were used. The incorporation of [^{3}H]thymidine by T cells cultured alone in the presence or absence of exogenous GM-CSF was < 300 cpm.

ing DC differentiated morphologically and retained their viability for several weeks (data not shown). No enhancement of DC differentiation was observed when IL-1, IL-3, or IL-4 was added simultaneously with GM-CSF at day 0 of culture (data not shown). These results suggest that a variety of interleukins have a relatively short-term impact on DC survival but do not induce DC morphologic differentiation or support their longer-term survival.

The question remained as to whether DC cultured in the presence of Tsn or GM-CSF for prolonged periods retained immune stimulatory activity. In preliminary experiments DC maintained for as long as 30 d in either Tsn or GM-CSF retained their ability to stimulate allogeneic T cells in the MLR, whereas DC cultured in medium alone lost all stimulatory activity. As shown in Fig. 5, DC but not monocytes/macrophages, require exogenous GM-CSF to maintain their immunostimulatory properties during prolonged culture. Indeed, the residual stimulatory activity of DC-enriched preparations remaining after 18 d of culture in the absence of exogenous GM-CSF may not be a function of DC since no cells with DC characteristics could be identified in such cultures. Fresh B cells failed to stimulate T cells in allogeneic MLRs and no stimulatory activity in the B cell fraction was induced during the 18-d culture with GM-CSF. GM-CSF added directly to MLRs between T cells and fresh allogeneic DC or fresh allogeneic macrophages had no effect on T cell proliferation (Fig. 5, *a* and *d*).

DC cultured in the presence of GM-CSF for 18 d also retained the ability to stimulate the proliferation of autologous T cells in autologous MLRs (Fig. 5 *c*). In contrast, despite their ability to stimulate allogeneic T cells (Fig. 5 *d*), neither fresh macrophages nor macrophages cultured in the presence or absence of GM-CSF had any stimulatory effect on autologous T cells.

As a general rule, DC isolated from a variety of tissues do not require exogenous cytokines to provide accessory cell/immune stimulatory function for T cells (reviewed in Steinman [15]). Mouse epidermal Langerhans cells, a type of DC, represent an exception to this rule. Despite their rich expression of class II MHC determinants, such cells fail to stimulate allogeneic T cells unless they are first exposed to GM-CSF (16, 17). After 1-3 d culture in GM-CSF, the accessory activity of Langerhans cells increases 10–30-fold, equaling the potent activity of splenic DC. IL-1 enhances the effect of GM-CSF but has no independent role in the maturation of Langerhans cells (16, 17). The effects of GM-CSF on human peripheral blood DC observed here—induction of morphologic changes as well as long-term viability—are clearly distinct from the pattern described for mouse epidermal Langerhans cells. More importantly, these findings indicate that GM-CSF can directly regulate the activity of functionally competent dendritic accessory cells. Since this cytokine is produced by a variety of cell types, including immunologically stimulated T cells, macrophages and endothelial cells (10, 11, 18), any or all of these cell popu-

lations can potentially be involved in the regulation of peripheral blood DC in vivo. In addition, it is possible that the effect of GM-CSF, on DC contributes to the clinical benefits observed in the initial therapeutic trials of recombinant GM-CSF in patients with cancer (19) and AIDS (20).

Acknowledgments

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X.   RELATED PROCEEDINGS APPENDIX

(None)

XI.   APPENDIX A: Chart of Support in Priority Application for Present Claims

APPENDIX A
Support in specification of priority application U.S. App. No. 07/861,612
for present claims of U.S. App. No. 09/073,596

(Note claims are grouped by dependency for ease of review)

Claim	Limitation	Support in specification of first-filed priority document U.S. App. No. 07/861,612 includes:
Claim 101 (cont'd next page)	An *in vitro* composition comprising mature dendritic cells expressing modified antigen and derived from an *in vitro* culture of an enriched and expanded population of proliferating dendritic cell precursors by a method comprising:	Abstract of the Disclosure, which states in part: "A method for producing proliferating cultures of dendritic cell precursors is provided. Also provided is a method for producing mature dendritic cells in culture from the proliferating dendritic cell precursors. The cultures of mature dendritic cells provide an effective means of producing novel T cell dependent antigens comprised of dendritic cell modified antigens or **dendritic cells pulsed with antigen which antigen is processed and expressed on the antigen-activated dendritic cell**. The novel antigens of the invention may be used as immunogens for vaccines or for the treatment of disease." Page 16, lines 2-6: "GM-CSF has surprisingly been found to promote the proliferation *in vitro* of precursor dendritic cells. **Cells are cultured in the presence of GM-CSF** at a concentration sufficient to promote the survival and proliferation of dendritic cell precursors." Also see p. 9, lines 25-28, "An object of this invention is to provide a method of culturing dendritic cell precursors *in vitro* so that they mature into mature dendritic cells suitable for use as immunogens or adjuvants when combined with an antigen." Original claim 36: "A composition comprising antigen-activated dendritic cells wherein dendritic cells prepared according to claim 17 are pulsed with an antigen and wherein the dendritic cells process the antigen to produce a modified antigen which is expressed by the dendritic cells."

| (claim 101, cont'd.) | providing a tissue source comprising dendritic cell precursors;

treating the tissue source comprising dendritic cell precursors to increase the proportion of dendritic cell precursors;

culturing the tissue source on a substrate in a **culture medium comprising GM-CSF** to obtain nonadherent cells and cell clusters;
subculturing the nonadherent cells and cell clusters to produce cell aggregates comprising proliferating dendritic cell precursors;
serially subculturing the cell aggregates one or more times to enrich the proportion of dendritic cell precursors; and
continuing to culture the dendritic cell precursors for a period of time to allow them to mature into mature dendritic cells;

culturing the dendritic cells *in vitro* in the presence of an antigen for a time sufficient to allow the antigen to bind to the dendritic cells, wherein the dendritic cells process the antigen to produce a modified antigen which is expressed by the dendritic cells. | Original claim 17: "A method of producing a population of mature dendritic cells from proliferating cell cultures comprising:
   a) providing a tissue source comprising dendritic cell precursors;
   b) treating the tissue source to obtain a population of cells suitable for culture *in vitro*;
     [see also p. 13, lines 16-18: "According to the method of the invention, the tissue source may be treated prior to culturing to enrich the proportion of dendritic precursor cells relative to other cell types."]

   c) culturing the tissue source on a substrate in a **culture medium comprising GM-CSF** to obtain nonadherent cells and cell clusters;
   d) subculturing the nonadherent cells and cell clusters to produce cell aggregates comprising proliferating dendritic cell precursors;
   e) serially subculturing the cell aggregates one or more time to enrich the proportion of dendritic cell precursors; and
   f) continuing to culture the dendritic cell precursors for a period of time sufficient to allow them to mature into mature dendritic cells."
     [see also page 7, line 22 through page 8, line 6, which recites the same steps set forth in original claim 17]
Original claim 36: "A composition comprising antigen-activated dendritic cells wherein dendritic cells prepared according to claim 17 are pulsed with an antigen and wherein the dendritic cells process the antigen to produce a modified antigen which is expressed by the dendritic cells."

   Also see p. 8, lines 15-19: "Another embodiment of the invention are antigen-activated dendritic cells prepared according to the method of the invention [in] which antigen-activated dendritic cells have been exposed to antigen and express modified antigen for presentation to and activation of T cells." |

(claim 101, cont'd.)		Also see p. 22, lines 10-20: "The antigen-activated dendritic cells of the invention are produced by exposing antigen, *in vitro*, to the dendritic cells prepared according to the method of the invention. Dendritic cells are plated in culture dishes and exposed to antigen in a sufficient amount and for a sufficient period of time to allow the antigen to bind to the dendritic cells." Also see p. 6, lines 7-14: "Dendritic cells bind and modify antigens in a manner such that the modified antigen when presented on the surface of the dendritic cell can activate T-cells to participate in the eventual production of antibodies. The modification of antigens by dendritic cells may, for example, include fragmenting a protein to produce peptides which have regions which specifically are capable of activating T-cells." Also see p. 21, line 30 through p. 22, line 5: "Foreign and autoantigens are processed by the dendritic cells of the invention to retain their immunogenic form. The immunogenic form implies processing the antigen through fragmentation to produce a form of the antigen that can be recognized by and stimulate T cells."
Claim 104	The composition according to claim 101, wherein the tissue source is blood.	*e.g.,* page 14, lines 5-19, beginning: "When blood is used as a tissue source...."
Claim 107	The composition according to claim 104, wherein the concentration of GM-CSF in the culture medium is about 30-100 U/ml.	*e.g.,* page 14, lines 10-12: "...cells from blood are cultured in the presence of GM-CSF at a concentration of between about 30 and 100 U/ml."
Claim 112	The composition according to claim 104, wherein the tissue source is treated to remove red blood cells.	*e.g.,* page 14, lines 7-19, beginning "According to the preferred method of the invention...." (line 7) and continuing, "Cells are pelleted and washed by centrifugation.... Platelets and red blood cells are depleted by suspending the cell pellet in a mixture of medium and ammonium chloride" (lines 10-13).
Claim 105	The composition according to claim 101, wherein the tissue source is bone marrow.	*e.g.,* page 13, lines 29-30: "When bone marrow is used as the tissue source...."

Claim 108	The composition according to claim 105, wherein the concentration of GM-CSF in the culture medium is about 500-1000 U/ml.	*e.g.*, page 16, lines 18-20: "Doses between about 500-1000 U/ml are preferred for cultures of cells obtained from marrow."
Claim 113	The composition according to claim 105, wherein the tissue source is treated to remove B cells and granulocytes.	*e.g.*, page 13, line 29 through page 14, line 4: "When bone marrow is used as the tissue source, it is preferred to remove B cells.... In addition, it is preferred to use procedures which minimize the number of granulocytes...."
Claim 106	The composition according to claim 101, wherein GM-CSF is present in the culture medium at a concentration of about 1 – 1000 U/ml.	*e.g.*, page 16, lines 9-10: "Preferably, the cells are cultured in the presence of between about 1 and 1000 U/ml of GM-CSF."
Claim 109	The composition according to claim 101, wherein the cell aggregates are blood derived and are subcultured from about one to five times.	*e.g.*, page 14, lines 5-19, beginning: "When blood is used as a tissue source...." and *e.g.*, page 18, line 26 through page 19, line 10, beginning "To further expand the population of dendritic cell, cell aggregates may be serially subcultured multiple times..." (page 18, lines 26-27) and ending, "[p]referably, cells can be serially subcultured...between about one to five times" (page 19, lines 8-10).
Claim 110	The composition according to claim 101, wherein the cell aggregates are subcultured one to five times.	*e.g.*, page 18, line 26 through page 19, line 10, beginning "To further expand the population of dendritic cell, cell aggregates may be serially subcultured multiple times..." (page 18, lines 26-27) and ending "[p]referably, cells can be serially subcultured...between about one to five times" (page 19, lines 8-10).
Claim 111	The composition according to claim 101, wherein the culture medium is selected from the group consisting of RPMI 1640, DMEM and α-MEM, and wherein the culture medium is supplemented with serum.	*e.g.*, page 15, lines 18-21, "Preferred medias include RPMI 1640, DMEM and α-MEM, with added amino acids and vitamins supplemented with an appropriate amount of serum...."
Claim 116	A pharmaceutical composition comprising a therapeutically effective amount of the composition according to claim 101.	*e.g.*, page 24, lines 4-11: "The activated dendritic cells...may be formulated for use as vaccines or pharmaceutical compositions with suitable carriers...."
Claim 99	The pharmaceutical composition according to claim 116, wherein the dendritic cells express an amount of the modified antigen to provide between about 1 to 100 micrograms of the modified antigen in said pharmaceutical composition.	*e.g.*, page 24, lines 7-13: "The vaccines or pharmaceutical compositions comprising the modified antigens or the antigen-activated dendritic cells of the invention would be administered in therapeutically effective amounts sufficient to elicit an immune response. Preferably, between about 1 to 100 micrograms of modified antigen, or its equivalent when bound to dendritic cells, should be administered per dose."

Claim 142	The composition according to claim 101, wherein the dendritic cell precursors are human.	*e.g.*, page 15, lines 13-26, beginning "[t]he growth medium for the cells at each step of the method of the invention should allow for the survival and proliferation of the precursor dendritic cells" (lines 13-15) and ending, "[c]ells from human tissue may also be cultured in medium supplemented with human serum...." (lines 24-26).
Claim 143	The composition according to claim 142, wherein the dendritic cell precursors are obtained from blood.	*e.g.*, page 14, lines 5-19, beginning: "When blood is used as a tissue source...."
Claim 144	The composition according to claim 142, wherein the dendritic cell precursors are obtained from bone marrow.	*e.g.*, page 13, lines 29-30: "When bone marrow is used as the tissue source...."
Claim 120	An *in vitro* composition comprising mature dendritic cells expressing a modified antigen and derived from an *in vitro* culture of a population of proliferating dendritic cell precursor cells by a method comprising culturing dendritic cell precursor cells in a culture medium comprising GM-CSF at a concentration sufficient to promote the survival and proliferation of dendritic cell precursors; serially subculturing the proliferating dendritic cell precursors at intervals which provide for the continued proliferation of said dendritic cell precursors; and exposing the cells to antigen *in vitro*, wherein the dendritic cells process the antigen to produce a modified antigen which is expressed by the dendritic cells.	As cited above for claim 101; see also page 18, lines 26-29: "To further expand the population of dendritic cells, cell aggregates may be serially subcultured multiple times at intervals which provide for the continued proliferation of dendritic cell precursors."

| Claim 145 | An *in vitro* composition comprising antigen-activated dendritic cells expressing modified antigens and derived from an *in vitro* culture of proliferating dendritic cell precursors by a method comprising:

    a) providing a tissue source comprising dendritic cell precursors;
    b) treating the tissue source to obtain a population of cells suitable for culture *in vitro*;
    c) culturing the tissue source on a substrate in a culture medium comprising GM-CSF to obtain nonadherent cells and cell clusters;
    d) subculturing the nonadherent cells and cell clusters to produce cell aggregates comprising proliferating dendritic cell precursors;
    e) serially subculturing the cell aggregates one or more time to enrich the proportion of dendritic cell precursors;
    f) continuing to culture the dendritic cell precursors for a period of time sufficient to allow them to mature into mature dendritic cells; and
    g) pulsing the dendritic cells with an antigen, wherein the dendritic cells process the antigen to produce a modified antigen which is expressed by the dendritic cells | (as cited above for claim 101; slightly different terminology was used)

(The term "pulsing" is synonymous with exposing the cell to a protein antigen, for example, as it is used in the specification on page 8, line 29 and in the Abstract.) |

XII.   APPENDIX B: Copy of Priority Application U.S. App. No. 07/861,612

PCT/US 93/05141

U.S. PATENT APPLICATION

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APPLICANT

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TITLE

METHOD FOR IN VITRO PROLIFERATION OF DENDRITIC CELL PRECURSORS AND THEIR USE TO PRODUCE IMMUNOGENS

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APR 20 1993

Date

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2016-4000

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES PATENT APPLICATION

Of: Ralph M. Steinman
Kayo Inaba
Gerold Schuler

For: METHOD FOR IN VITRO PROLIFERATION
OF DENDRITIC CELL PRECURSORS AND THEIR
USE TO PRODUCE IMMUNOGENS

4

5   TECHNICAL FIELD OF THE INVENTION

6   This invention relates to a method of culturing cells of the

7   immune system. In particular a method is provided for culturing

8   proliferating dendritic cell precursors and for their maturation

9   _in vitro_ to mature dendritic cells. This invention also relates

10 to dendritic cell modified antigens which are T cell dependent,

11 the method of making them, and their use as immunogens.

12 Vaccines, methods of immunizing animals and humans using the

13 mature dendritic cells of the invention, and the modified

14 antigens are also described.

15

16   BACKGROUND OF THE INVENTION

17   The immune system contains a system of dendritic cells that

18 is specialized to present antigens and initiate several T-

19 dependent immune responses. Dendritic cells are distributed

20 widely throughout the body in various tissues. The distribution

21 of dendritic cells has been reviewed in (1). Dendritic cells are

22 found in nonlymphoid organs either close to body surfaces, as in

23 the skin and airways, or in interstitial regions of organs like

24 heart and liver. Dendritic cells, possibly under the control of

25 the cytokine granulocyte macrophage colony-stimulating factor,

26 (hereinafter GM-CSF), can undergo a maturation process that does

27 not entail cell proliferation (2,3). Initially, the cells

28 process and present antigens most likely on abundant, newly

29 synthesized MHC class II molecules, and then strong accessory and

30 cell-cell adhesion functions are acquired (4-7). Dendritic cells

                              - 2 -

1   can migrate via the blood and lymph to lymphoid org.ns (8-10).

2   There, presumably as the "interdigitating" cells of the T-area

3   (8,11-13), antigens can be presented to T cells in the

4   recirculating pool (14). However, little is known about the

5   progenitors of dendritic cells in the different compartments

6   outlined above.

7   The efficacy of dendritic cells in delivering antigens in

8   such a way that a strong immune response ensues i.e.,

9   "immunogenicity", is widely acknowledged, but the use of these

10   cells is hampered by the fact that there are very few in any

11   given organ. In human blood, for example, about 0.1% of the

12   white cells are dendritic cells (25) and these have not been

13   induced to grow until this time. While dendritic cells can

14   process foreign antigens into peptides that immunologically

15   active T cells must recognize (4,6,7,14) i.e., dendritic cells

16   accomplish the phenomenon of "antigen presentation", the low

17   numbers of dendritic cells prohibits their use in identifying

18   immunogenic peptides.

19   Dendritic cells in spleen (15) and afferent lymph (16,17)

20   are not in the cell cycle but arise from a proliferating

21   precursor. Ultimately, dendritic cells emanate from the bone

22   marrow (15,16,18,19), yet it has been difficult to generate these

23   cells in culture except for two reports describing their

24   formation in small numbers (20,21). Although a bone marrow

25   precursor cell has been reported, conditions have not been

26   reported that direct its proliferation in culture. Steinman, R.

27   "The Dendritic Cell System and Its Role In Immunogenicity", Am.

28   Rev. Immunol., 9:271-96 (1991). The use of cell surface markers

29   to enrich bone marrow dendritic cell precursors has been reported

30   to result in only modest increases because the markers are also

1 expressed by numerous bone marrow cells. Bowers, W.E. and

2 Goodell, "Dendritic Cell Ontogeny" Res. Immunol. 140:880-883

3 (1989).

4   Relatively small numbers of dendritic cells have also been

5 cultured from blood. Vakkila J. et al. "Human Peripheral blood-

6 derived dendritic cells do not produce interleukin 1α,

7 interleukin 1β, or interleukin 6" Scand. J. Immunol. 31:345-352

8 (1990); Van Voorhis W.C. et al., "Human Dendritic Cells", J.Exp.

9 Med., 1172-1187 (1982). However, the presence in blood of

10 dendritic cell precursors has not been reported and as recently

11 as 1989 the relationship between blood dendritic cells and mature

12 dendritic cells in other tissues was uncertain. Furthermore, it

13 was recognized that dendritic cells are "rare and difficult to

14 isolate and have not as yet been shown to give rise to DC

15 [dendritic cells] in peripheral tissues." MacPherson G.G.

16 "Lymphoid Dendritic cells: Their life history and roles in immune

17 responses", Res. Immunol. 140:877-926 (1989).

18   Granulocyte/macrophage colony-stimulating factor (GM-CSF) is

19 a factor which modulates the maturation and function of dendritic

20 cells. Witmer-Pack et al, "Granulocyte/macrophage colony-

21 stimulating factor is essential for the viability and function of

22 cultured murine epidermal Langerhans cells". J.Exp.Med.

23 166:1484-1498 (1987). Heufler C. et al., "Granulocyte/macrophage

24 colony-stimulating factor and interleukin 1 mediate the

25 maturation of murine epidermal Langerhans cells into potent

26 immunostimulatory dendritic cells", J. Exp. Med. 167:700-705

27 (1988). GM-CSF stimulated maturation of dendritic cells in vitro

28 suggests that the presence of GM-CSF in a culture of dendritic

29 cell precursors would mediate maturation into immunologically

30 active cells, but the important goal of achieving extensive

- 4 -

1 dendritic cell growth has yet to be solved.

2   T-dependent immune responses are characterized by the

3 activation of T-helper cells in the production of antibody by B

4 cells. An advantage of T-dependent over T-independent immune

5 responses is that the T-dependent responses have memory, i.e.

6 cells remain primed to respond to antigen with rapid production

7 of antibody even in the absence of antigen and the immune

8 response is therefore "boostable". T-independent immune

9 responses are, in contrast, relatively poor in children and lack

10 a booster response when a T-independent antigen is repeatedly

11 administered. The immunologic memory of T cells likely reflects

12 two consequences of the first, "primary" or "sensitizing" limb of

13 the immune response: (a) an expanded number of antigen-specific T

14 cells that grow in response to antigen-bearing dendritic cells,

15 and (b) the enhanced functional properties of individual T cells

16 that occurs after dendritic cell priming [Inaba et al., Resting

17 and sensitized T lymphocytes exhibit distinct stimulatory

18 (antigen presenting cell) requirements for growth and lymphokine

19 release; J.Exp.Med. 160:868-876 (1984); Inaba and Steinman,

20 "Protein-specific helper T lymphocyte formation initiated by

21 dendritic cells", Science 229: 475-479 (1985); Inaba et al.,

22 "Properties of memory T lymphocytes isolated from the mixed

23 leukocyte reaction", Proc.Natl.Acad.Sci. 82:7686-7690 (1985)].

24   Certain types of antigens characteristically elicit T-cell

25 dependent antibody responses whereas others elicit a T-cell

26 independent response. For example, polysaccharides generally

27 elicit a T-cell independent immune response. There is no memory

28 response and therefore no protection to subsequent infection with

29 the polysaccharide antigen. Proteins, however, do elicit a T-

30 cell dependent response in infants. The development of conjugate

- 5 -

1   vaccines containing a polysaccharide covalently coupled to a

2   protein converts the polysaccharide T-independent response to a

3   T-dependent response. Unfortunately, little is known concerning

4   the sites on proteins which confer their T-cell dependent

5   character, therefore hampering the design of more specific

6   immunogens.

7   As stated above, dendritic cells play a crucial role in the

8   initiation of T-cell dependent responses. Dendritic cells bind

9   and modify antigens in a manner such that the modified antigen

10 when presented on the surface of the dendritic cell can activate

11 T-cells to participate in the eventual production of antibodies.

12 The modification of antigens by dendritic cells may, for example,

13 include fragmenting a protein to produce peptides which have

14 regions which specifically are capable of activating T-cells.

15   The events whereby cells fragment antigens into peptides,

16 and then present these peptides in association with products of

17 the major histocompatibility complex, are termed "antigen

18 presentation." Dendritic cells prove to be specialized antigen

19 presenting cells in the immune response of whole animals (14,31).

20 Again however, the ability to use dendritic cells to identify and

21 extract the immunogenic peptides is hampered by the small numbers

22 of these specialized antigen presenting cells.

23   Injection of dendritic cells pulsed with pathogenic

24 lymphocytes into mammals to elicit an active immune response

25 against lymphoma is the subject of PCT patent application WO

26 91/13632. In addition, Francotte and Urbain, PNAS 82:8149 (1985)

27 reported that mouse dendritic cells, pulsed in vitro with virus

28 and injected back into mice, enhances the primary response and

29 the secondary response to the virus. Neither the report by

30 Francotte and Urbain and patent application WO 91/13632 provide a

- 6 -

1 practical method of using dendritic cells as an adjuvant to

2 activate the immune response because both of these methods depend

3 on dendritic cells obtained from spleen, an impractical source of

4 cells for most therapies or immunization procedures. In

5 addition, neither report provides a method to obtain dendritic

6 cells in sufficient quantities to be clinically useful.

7

8   SUMMARY OF THE INVENTION

9   This invention claims a method of producing a population of

10 dendritic cell precursors from proliferating cell cultures. The

11 method comprises (a) providing a tissue source comprising

12 dendritic cell precursors; (b) treating the tissue source to

13 obtain a population of cells suitable for culture _in vitro_; (c)

14 culturing the tissue source on a substrate in a culture medium

15 comprising GM-CSF, or a biologically active derivative of GM-CSF,

16 to obtain proliferating nonadherent cells and cell clusters; (d)

17 subculturing the nonadherent cells and cell clusters to produce

18 cell aggregates comprising proliferating dendritic cell

19 precursors; and (e) serially subculturing the cell aggregates one

20 or more times to enrich the proportion of dendritic cell

21 precursors.

22   This invention also claims a method of producing _in vitro_

23 mature dendritic cells from proliferating cell cultures. The

24 method comprises (a) providing a tissue source comprising

25 dendritic cell precursor cells; (b) treating the tissue source to

26 obtain a population of cells suitable for culture _in vitro_; (c)

27 culturing the tissue source on a substrate in a culture medium

28 comprising GM-CSF, or a biologically active derivative of GM-CSF,

29 to obtain non-adherent cells and cell clusters; (d) subculturing

30 the nonadherent cells and cell clusters to produce cell

- 7 -

1 aggregates comprising proliferating dendritic cell precursors;

2 (e) serially subculturing the cell aggregates one or more times

3 to enrich the proportion of dendritic cell precursors; and (f)

4 continuing to culture the dendritic cell precursors for a period

5 of time sufficient to allow them to mature into mature dendritic

6 cells.

7 To reduce the proportion of non-dendritic precursor cells,

8 the tissue source may be pre-treated prior to culturing the

9 tissue source on a substrate to obtain the non-adherent cells or

10 during the early stages of the culture. Preferred tissue sources

11 for the practice of the invention are bone marrow and, in

12 particular, blood.

13 The dendritic cell precursors and dendritic cells prepared

14 according to the method of this invention are also claimed.

15 Another embodiment of the invention are antigen-activated

16 dendritic cells prepared according to the method of the invention

17 which antigen-activated dendritic cells have been exposed to

18 antigen and express modified antigens for presentation to and

19 activation of T cells.

20 This invention also provides novel antigens which are

21 produced by exposing an antigen to cultures of dendritic cells

22 prepared according to the method of the invention which antigen

23 is modified by the dendritic cells to produce modified antigens

24 which are immunogenic fragments of the unmodified or native

25 antigen and which fragments activate T cells.

26 These novel antigens may be used to immunize animals and

27 humans to prevent or treat disease.

28 In a further embodiment, the invention claims self-peptide

29 antigens produced by pulsing the dendritic cells of the invention

30 with a protein to which an individual has developed an immune

- 8 -

1 response and extracting the relevant self-peptide or autoantigen.

2 This invention also claims a method of treating autoimmune

3 diseases by treating an individual with therapeutically effective

4 amounts of self-peptides produced according to the method of the

5 invention to induce tolerance to the self-proteins.

6 The treatment of autoimmune diseases comprising

7 administering to an individual in need of treatment a

8 therapeutically effective amount of antigen-activated dendritic

9 cells where the antigen is a self-protein or autoantigen is also

10 claimed.

11 The use of the compositions and methods of the invention to

12 treat autoimmune diseases selected from the group of juvenile

13 diabetes and multiple sclerosis is also claimed.

14 This invention also provides a method for providing an

15 antigen to a host comprising exposing an antigen to a culture of

16 dendritic cells prepared according to the method of this

17 invention to produce antigen-activated dendritic cells followed

18 by inoculating the host with the antigen-activated dendritic

19 cells.

20 Vaccines comprised of any of the antigens or antigen-

21 activated dendritic cells described above are also claimed as are

22 the methods of immunizing against disease in humans or animals

23 comprising administering of any of the compositions of the

24 invention.

25 An object of this invention is to provide a method of

26 culturing dendritic cell precursors in vitro so that they mature

27 into mature dendritic cells suitable for use as immunogens or

28 adjuvants when combined with an antigen.

29 Another object of this invention is to provide a convenient

30 and practical source of sufficient quantities of dendritic cells

- 9 -

1   to be useful in the treatment or prevention of disease.

2   Another object of this invention is to provide novel

3   immunogens comprising the dendritic cells of this invention which

4   have been exposed to antigen and express modified antigen on

5   their surface.

6   Another object of this invention is to provide antigens

7   which have been modified through their exposure to dendritic

8   cells and which modified antigens are effective as T-cell

9   dependent antigens.

10   A further objective of the invention is to provide a method

11   of immunizing individuals with T-cell dependent antigens for the

12   prevention and treatment of disease.

13

14

15   FIGURE LEGENDS

16   Fig. 1.   Flow plan for inducing dendritic cell "colonies."

17

18   Fig. 2   Phase contrast micrographs of dendritic cells

19   developing in vitro.

20   A.   Low power view of a monolayer covered with aggregates

21   of proliferating dendritic cell progenitors [day 17

22   culture].

23   B.   Higher power view of typical dendritic cells [arrows]

24   released from the aggregates.

25

26   Fig. 3   Immunoperoxidase labeling of a culture transferred

27   after 2-3 wks to show some of the typical phenotypic features of

28   released dendritic cell progeny. The primary antibodies are B21-

29   2 anti-MHC class II [ATCC #TIB 229]; M1/69 anti-heat stable

30   antigen [HSA, ATCC #TIB 125]; F4/80 anti-macrophage [MAC, ATCC

- 10 -

1 #HB 198]; NLDC 145 (13) anti-interdigitating cell [IDC, kindly

2 provided by Dr. G. Kraal, Free University, Amsterdam]; RB6 anti-

3 granulocyte [an example of a rare granulocyte is shown; GRAN];

4 and 2A1, a mAb that reacts primarily with granules within the

5 cytoplasm of cultured dendritic cells in vitro [in preparation],

6 and interdigitating cells in situ. 600X

7

8 Fig. 4   FACS analyses of dendritic cells released from

9 proliferating aggregates. Several mAbs summarized elsewhere

10 (23,24,28) are shown. Except for MHC class I and II products,

11 the phenotype of the released cells is homogeneous. The staining

12 with no primary mAb was identical to RB6 and RA3.

13

14 Fig. 5   FACS analyses of dendritic cells that could be

15 dislodged by Pasteur pipetting of proliferating aggregates, and

16 dendritic cells released spontaneously in culture. The mAb are:

17 M1/42 anti-MHC class I [ATCC # TIB 126]; NLDC145 anti-

18 interdigitating cell (13); M5/114 anti-MHC class II [ATCC # TIB

19 120]; 33D1 anti-dendritic cell [ATCC # TIB 227]; B5-5 anti-thy-1.

20 The staining with anti-MHC mAbs is bimodal, but the released cell

21 fraction of dendritic cells is richest in expression of MHC class

22 I and II.

23

24 Fig. 6   ³H-TdR pulse & pulse-chase labeling of proliferating

25 dendritic cell aggregates and their progeny respectively. The

26 left and middle panels illustrate pulse labeling of aggregates [1

27 uCi/ml for 2h] at about 2 wks of culture. Prior to dipping in

28 photographic emulsion the cytospins were stained with an

29 immunoperoxidase method to identify the 2A1 anti-dendritic cell

30 granule antigen [left] or the NLDC145 interdigitating cell

- 11 -

1 antigen (middle). The cells that label for these antigens [black

2 arrows] are not in S-phase [white arrows]. The right panel

3 illustrates a pulse chase label [0.2 uCi/ml for 16h, chase for 2

4 days] of aggregates that were isolated by velocity sedimentation

5 and returned to culture to form large numbers of released

6 progeny. In contrast to the left panel, the 3H-TdR labeled cells

7 express the 2A1 granule antigen characteristic of mature

8 dendritic cells [black arrows].

9 425X.

10

11 Fig 7.   MLR stimulating activity of populations isolated from

12 the GM-CSF stimulated mouse blood cultures [see text].

13

14 Fig. 8   Homing activity of dendritic cells released from

15 proliferating cell aggregates. The dendritic cells were tagged

16 with carboxyfluorescein and as a result could be labeled in these

17 cryostat sections with a peroxidase conjugated anti-FITC antibody

18 plus diaminobenzidine [brown color]. The sections were then

19 double labeled with mAb's to B cells [left; RA3-6B2.1 anti-B220]

20 or T cells [right; 53-6.7 anti-CD8; ATCC # TIB 105]. Note that

21 the dendritic cells [brown profiles] homed to the deep cortex of

22 the lymph node which are the sites containing most of the

23 recirculating T cells as well as interdigitating cells. The

24 upper low power views are at 43X, and the lower higher power

25 micrographs are at 170X.

26

27   DETAILED DESCRIPTION OF THE INVENTION

28   This invention relates to a method of producing cultures of

29 proliferating dendritic cell precursors which mature in vitro to

30 mature dendritic cells. The dendritic cells produced according

1   to the method of the invention may be produced in amounts

2   suitable for various immunological interventions for the

3   prevention and treatment of disease.

4   The starting material for the method of producing dendritic

5   cell precursors and mature dendritic cells is a tissue source

6   comprising dendritic cell precursors which precursor cells are

7   capable of maturing in vitro into dendritic cells when treated

8   according to the method of the invention. Such precursor cells

9   are nonadherent and typically do not label with mAb markers found

10 on mature dendritic cells such as 2A1 and NLDC145. Preferably

11 such tissue sources are spleen, afferent lymph, bone marrow and

12 blood. More preferred tissue sources are bone marrow and blood.

13 Blood is also a preferred tissue source of precursor cells

14 because it is easily accessible and could be obtained in

15 relatively large quantities.

16   According to the method of the invention, the tissue source

17 may be treated prior to culturing to enrich the proportion of

18 dendritic precursor cells relative to other cell types. Such

19 pretreatment may also remove cells which may compete with the

20 proliferation of dendritic precursor cells or inhibit their

21 proliferation or survival. Pretreatment may also be used to make

22 the tissue source more suitable for in vitro culture. The method

23 of treatment will likely be tissue specific depending on the

24 particular tissue source. For example, spleen or bone marrow if

25 used as a tissue source would first be treated so as to obtain

26 single cells followed by suitable cell separation techniques to

27 separate leukocytes from other cell types. Treatment of blood

28 would involve cell separation techniques to separate leukocytes

29 from other cells types. When bone marrow is used as the tissue

30 source, it is preferred to remove B cells prior to culturing the

- 13 -

1   cells. In addition, it is preferred to use procedures which

2   minimize the number of granulocytes in the bone marrow cultures

3   because the granulocytes have a tendency to overgrow the cultures

4   and compete for available GM-CSF.

5   When blood is used as a tissue source, blood leukocytes may

6   be obtained using conventional methods which maintain their

7   viability. According to the preferred method of the invention,

8   blood is diluted into medium (preferably RPMI) containing heparin

9   (about 100 U/ml) or other suitable anticoagulant. The volume of

10   blood to medium is about 1 to 1. Cells are pelleted and washed

11   by centrifugation of the blood in medium at about 1000 rpm (150g)

12   at 4°C. Platelets and red blood cells are depleted by suspending

13   the cell pellet in a mixture of medium and ammonium chloride.

14   Preferably the mixture of medium to ammonium chloride (final

15   concentration 0.839 percent) is about 1:1 by volume. Cells are

16   pelleted by centrifugation and washed about 2 more times in the

17   medium-ammonium chloride mixture, or until a population of

18   leukocytes, substantially free of platelets and red blood cells,

19   is obtained.

20   Any isotonic solution commonly used in tissue culture may be

21   used as the medium for separating blood leukocytes from platelets

22   and red blood cells. Examples of such isotonic solutions are

23   phosphate buffered saline, Hanks balanced salt solution, or

24   complete growth mediums including for example RPMI 1640. RPMI

25   1640 is preferred.

26   Cells obtained from treatment of the tissue source are

27   cultured to form a primary culture on an appropriate substrate in

28   a culture medium supplemented with GM-CSF or a GM-CSF derivative

29   protein or peptide having an amino acid sequence which sequence

30   maintains biologic activity typical of GM-CSF. The appropriate

- 14 -

1   substrate may be any tissue culture compatible surface to which

2   cells may adhere. Preferably, the substrate is commercial

3   plastic treated for use in tissue culture. Examples include

4   various flasks, roller bottles, petri dishes and multi-well

5   containing plates made for use in tissue culture. Surfaces

6   treated with a substance, for example collagen or poly-L-lysine,

7   or antibodies specific for a particular cell type to promote cell

8   adhesion may also be used provided they allow for the

9   differential attachment of cells as described below. Cells are

10 preferably plated at an initial cell density of about 7.5 X 10^5

11 cells per cm^2. At this dose, the surface is not fully covered by

12 cells, but there are no big spaces (2-3 cell diameters) either.

13   The growth medium for the cells at each step of the method

14 of the invention should allow for the survival and proliferation

15 of the precursor dendritic cells. Any growth medium typically

16 used to culture cells may be used according to the method of the

17 invention provided the medium is supplemented with GM-CSF.

18 Preferred medias include RPMI 1640, DMEM and α-MEM, with added

19 amino acids and vitamins supplemented with an appropriate amount

20 of serum or a defined set of hormones and an amount of GM-CSF

21 sufficient to promote precursor dendritic cell proliferation.

22 RPMI 1640 supplemented with 5% fetal calf serum (FCS) and GM-CSF

23 is preferred. Cells may be selected or adapted to grow in other

24 serums and at other concentrations of serum. Cells from human

25 tissue may also be cultured in medium supplemented with human

26 serum rather than FCS. Medias may contain antibiotics to

27 minimize bacteria infection of the cultures. Penicillin,

28 streptomycin or gentamicin or combinations containing them are

29 preferred. The medium, or a portion of the medium, in which the

30 cells are cultured should be periodically replenished to provide

- 15 -

1   fresh nutrients including GM-CSF.

2   GM-CSF has surprisingly been found to promote the

3   proliferation in vitro of precursor dendritic cells. Cells are

4   cultured in the presence of GM-CSF at a concentration sufficient

5   to promote the survival and proliferation of dendritic cell

6   precursors. The dose depends on the amount of competition from

7   other cells (especially macrophages and granulocytes) for the GM-

8   CSF, or to the presence of GM-CSF inactivators in the cell

9   population. Preferably, the cells are cultured in the presence

10 of between about 1 and 1000 U/ml of GM-CSF. More preferably

11 cells from blood are cultured in the presence of GM-CSF at a

12 concentration of between about 30 and 100 U/ml. This dose has

13 been found to be necessary and sufficient for maximal responses

14 by cells obtained from mouse blood. Most preferably, cells are

15 cultured in the presence of GM-CSF at a concentration of about 30

16 U/ml. Cells from bone marrow require higher concentrations of

17 GM-CSF because of the presence of large numbers of proliferating

18 granulocytes which compete for the available GM-CSF. Doses

19 between about 500-1000 U/ml are preferred for cultures of cells

20 obtained from marrow.

21   When mouse blood leukocytes are cultured in GM-CSF at 30

22 U/ml, the cultures develop a large number of aggregates from

23 which typical dendritic cells are eventually released. In the

24 absence of GM-CSF, no colonies develop. Cytologic criteria may

25 be used to initially detect the dendritic cells which

26 characteristically extend large, sheet-like processes or veils

27 (25-27).

28   GM-CSF may be isolated from natural sources, produced using

29 recombinant DNA techniques or prepared by chemical synthesis.

30 As used herein, GM-CSF includes GM-CSF produced by any method and

- 16 -

1 from any species. "GM-CSF" is defined herein as any bioactive

2 analog, fragment or derivative of the naturally occurring

3 (native) GM-CSF. Such fragments or derivative forms of GM-CSF

4 should also promote the proliferation in culture of a dendritic

5 cell precursors. In addition GM-CSF peptides having biologic

6 activity can be identified by their ability to bind GM-CSF

7 receptors on appropriate cell types.

8   The primary cultures from the tissue source are allowed to

9 incubate at about 37°C under standard tissue culture conditions

10 of humidity and pH until a population of cells has adhered to the

11 substrate sufficiently to allow for the separation of nonadherent

12 cells. The dendritic cell precursor in blood initially is

13 nonadherent to plastic, in contrast to monocytes, so that the

14 precursors can be separated after overnight culture. Monocytes

15 and fibroblasts are believed to comprise the majority of adherent

16 cells and usually adhere to the substrate within about 6 to about

17 24 hours. Preferably nonadherent cells are separated from

18 adherent cells between about 8 to 16 hours. Most preferably

19 nonadherent cells are separated at about 12 hours. Any method

20 which does not dislodge significant quantities of adherent cells

21 may be used to separate the adherent from nonadherent cells.

22 Preferably, the cells are dislodged by simple shaking or

23 pipetting. Pipetting is most preferred.

24   The nonadherent cells from the primary culture are

25 subcultured by transferring them to new culture flasks at a

26 density sufficient to allow for survival of the cells and which

27 results in the development over time of clusters of growing cells

28 that are loosely attached to the culture surface or to the firmly

29 adherent cells on the surface. These clusters are the nidus of

30 proliferating dendritic cell precursors. As used herein "culture

- 17 -

1 flasks" refers to any vessel suitable for culturing cells. It is

2 desirable to subculture all of the nonadherent cells from the

3 primary culture at a density of between about 2 X 10^5 cells and

4 5 X 10^5 cells per cm^2. Preferably at about 2.5 X 10^5 per cm^2.

5 Cells are incubated for a sufficient time to allow the surface of

6 the culture dish to become covered with a monolayer of tightly

7 adherent cells including macrophages and fibroblasts affixed to

8 which are aggregates of nonadherent cells. At this time, any

9 nonadherent cells are removed from the wells, and the cellular

10 aggregates are dislodged for subculturing. Preferably the cells

11 from the aggregates are subcultured after about 10 days or when

12 the number of aggregated cells per cm^2 reaches about 3 to 4 X

13 10^5.

14   For serially subculturing the aggregated cells, the

15 aggregated cells are dislodged from the adherent cells and the

16 aggregated cells are subcultured on a total surface area of

17 preferably between about 2 to 5 times that of the surface area of

18 the parent culture. More preferably the cells are subcultured on

19 a surface area that is about 3 times the surface area of the

20 parent culture. Cells having sheet-like processes typical of

21 dendritic cells appear in the culture at about 4-7 days. Between

22 about day 10 and day 17 of culture the number of single cells

23 that can be recovered from a given surface area doubles. Both

24 dendritic cell precursors and mature dendritic cells are present

25 in the aggregates.

26   To further expand the population of dendritic cells, cell

27 aggregates may be serially subcultured multiple times at

28 intervals which provide for the continued proliferation of

29 dendritic cell precursors. Preferably, aggregates are

30 subcultured prior to the release into the medium of a majority of

1 cells having the dendritic cell morphology, for example between
2 about 3 and 30 days. More preferably aggregates of cells are
3 subcultured between about 10 to 25 days in culture, and most
4 preferably at 20 days. The number of times the cells are
5 serially subcultured depends on the number of cells desired, the
6 viability of the cells, and the capacity of the cultures to
7 continue to produce cell aggregates from which dendritic cells
8 are released. Preferably, cells can be serially subcultured for
9 between about 1 to 2 months from when the nonadherent cells were
10 subcultured or between about one to five times. More preferably
11 cells are serially subcultured about two to three times. Most
12 preferably cells are serially subcultured twice.

13   According to a preferred method, to serially subculture the
14 cells of the primary and subsequent cultures, cells are dislodged
15 by pipetting most of the aggregates of growing dendritic cells as
16 well as some cells in the monolayer of growing macrophages and
17 fibroblasts. Pipetting usually disrupts the aggregates,
18 particularly the peripheral cells of the aggregates which are
19 more mature. With time in culture, e.g., at 2 weeks, the
20 aggregates of the growing dendritic cells become more stable and
21 it is possible to dislodge the aggregates for separation by 1g
22 sedimentation.

23   Typically the contents of 5 16 mm wells are applied to a 6
24 ml column of 50% FCS -RPMI 1640 in a 15 ml conical tube
25 [Sarstedt, 62.553.002 PS]. After at least 20 min, the applied
26 medium and top 1 ml of the column are removed. RPMI is added,
27 the aggregates are pelleted at 1000 rpm at 4° for 5 min, and the
28 cells are suspended gently for subculture in fresh medium.

29   Various techniques may be used to identify the cells present
30 in the cultures. These techniques may include analysis of

- 19 -

1 morphology, detecting cell type specific antigens with monoclonal

2 antibodies, identifying proliferating cells using tritiated

3 thymidine autoradiography, assaying mixed leukocyte reactions,

4 and demonstrating dendritic cell homing.

5 The dendritic cells besides being identified by their

6 stellate shape may also be identified by detecting their

7 expression of specific antigens using monoclonal antibodies.

8 A panel of monoclonal antibodies may be used to identify and

9 characterize the cells in the GM-CSF expanded cultures. The

10 monoclonal antibodies are reviewed elsewhere (23,24 which are

11 incorporated herein by reference).

12 Among the specific monoclonal antibodies suitable for

13 identifying dendritic cells are: 1) those which bind to the MHC

14 class I antigen (M1/42 anti-MHC class I [ATCC # TIB 126]); 2)

15 those which bind to the MHC class II antigen (B21-2 anti-MHC

16 class II [ATCC # TIB 229]; M5/114 anti-MHC class II [ATCC # TIB

17 120]); 3) those which bind to heat stable antigen (M1/69 anti-

18 heat stable antigen [HSA, ATCC #TIB 125]); 4) 33D1 anti-dendritic

19 cell antibodies [ATCC # TIB 227] those which bind to the

20 interdigitating cell antigen (NLDC145 anti-interdigitating cell

21 (13); and 6) those which bind to antigens in granules in the

22 perinuclear region of mature dendritic cells (monoclonal

23 antibodies 2A1 and M342, Agger et al.). Other antigens which are

24 expressed by the dendritic cells of the invention and which may

25 be used to identify mature dendritic cells are CD44 (identified

26 with monoclonal antibody M1/69), and CD11b (identified with

27 monoclonal antibody M1/70. The M1/69, M1/70, M1/42 monoclonal

28 antibodies are described in Monoclonal antibodies, NY, Plenum

29 1980, ed. R. Kennett et al. pages 185-217 which is incorporated

30 herein by reference.

- 20 -

To identify and phenotype the proliferating cells and their
progeny, cultures may be labelled with tritiated thymidine to
identify the cells in the S phase of mitosis. In addition to
labelling the cells with a mitotic label, cells may also be co-
labelled with monoclonal antibodies to determine when markers
associated with mature dendritic cells are expressed.

Another index of dendritic cell maturity is the ability of
mature dendritic cells to stimulate the proliferation of T-cells
in the mixed leukocyte reaction (MLR). The ability of dendritic
cells to migrate to lymph nodes, i.e., dendritic cell homing is
another index of dendritic cell maturation which may be used to
assess the maturity of the cells in culture.

The criteria that have become evident for identifying
dendritic precursor cells according to the invention enables the
identification of proliferating progenitors of dendritic cells in
other organs. It is known that proliferating precursors give
rise to the rapidly turning over populations of dendritic cells
in spleen (15) and afferent lymph (16). The proliferation of
leukocytes [other than T cells] occurs in the bone marrow, but it
may be that for dendritic cells, the marrow also seeds the blood
and other tissues with progenitors which then proliferate
extensively as shown here. By being able to prepare the
otherwise trace dendritic cell in large numbers, other previously
unexplored areas of dendritic cell function may be determined.
Specifically, growing dendritic cells will facilitate molecular
and clinical studies on the mechanism of action of these APCs,
including their capacities to capture and retain antigens in an
immunogenic form (4,6,14) and act as adjuvants for the generation
of immunity in vivo (1,14,31).

Foreign and autoantigens are processed by the dendritic

- 21 -

1 cells of the invention to retain their immunogenic form. The

2 immunogenic form of the antigen implies retaining the native

3 epitopes of immunogenic determinants on the antigen while

4 processing the antigen through fragmentation to produce a form of

5 the antigen that can be recognized by and stimulate T cells.

6 Preferably, such foreign or autoantigens are proteins which are

7 processed into peptides by the dendritic cells. The relevant

8 peptides which are produced by the dendritic cells may be

9 extracted and purified for use as immunogens.

10 The antigen-activated dendritic cells of the invention are

11 produced by exposing antigen, in vitro, to the dendritic cells

12 prepared according to the method of the invention. Dendritic

13 cells are plated in culture dishes and exposed to antigen in a

14 sufficient amount and for a sufficient period of time to allow

15 the antigen to bind to the dendritic cells. The amount and time

16 necessary to achieve binding of the antigen to the dendritic

17 cells may be determined by immunoassay or binding assay. Other

18 methods known to those of skill in the art may be used to detect

19 the presence of antigen on the dendritic cells following their

20 exposure to antigen.

21 The present invention provides for the first time a method

22 of obtaining dendritic cells in sufficient quantities to be used

23 to treat or immunize animals or humans with dendritic cells which

24 have been activated with antigens. In addition dendritic cells

25 may be obtained in sufficient quantities to be useful as reagents

26 to modify antigens in a manner to make the antigens more

27 effective as T-cell dependent antigens.

28 To use antigen-activated dendritic cells as a therapeutic or

29 immunogen the antigen-activated dendritic cells are injected by

30 any method which elicits an immune response into a syngeneic

- 22 -

1   arimal or human. Preferably, dendritic cells are injected back

2   into the same animal or human from whom the source tissue was

3   .obtained. The injection site may be subcutaneous,

4   intraperitoneal, intramuscular, or intravenous. The number of

5   antigen-activated dendritic cells reinjected back into the animal

6   or human in need of treatment may vary depending on _inter alia_,

7   the antigen and size of the individual. A key feature in the

8   function of dendritic cells _in situ_ is the capacity to migrate or

9   home to the T-dependent regions of lymphoid tissues, where the

10   dendritic cells would be in an optimal position to select the

11   requisite antigen-reactive T cells from the pool of recirculating

12   quiescent lymphocytes and thereby initiate the T-dependent

13   response.

14   The novel antigens of the invention are prepared by

15   combining substances to be modified or other antigens with the

16   dendritic cells prepared according to the method of the

17   invention. The dendritic cells process or modify antigens in a

18   manner which promotes the stimulation of T-cells by the processed

19   or modified antigens. Such dendritic cell modified antigens are

20   advantageous because they can be more specific and have fewer

21   undesirable epitopes than non-modified T-dependent antigens. The

22   dendritic cell modified antigens may be purified by standard

23   biochemical methods. For example, it is known to use antibodies

24   to products of the major histocompatibility complex (MHC) to

25   select MHC-antigenic peptide complexes and then to elute the

26   requisite processed peptides with acid [Rudensky et al., _Nature_

27   353:622-7 (1991); Hunt et al., _Science_ 255: 1261-3 (1992) which

28   are incorporated herein by reference].

29   Antigen-activated dendritic cells and dendritic cell

30   modified antigens may both be used to elicit an immune response

1 against an antigen. The activated dendritic cells or modified

2 antigens may by used as vaccines to prevent future infection or

3 may be used to activate the immune system to treat ongoing

4 disease. The activated dendritic cells or modified antigens may

5 be formulated for use as vaccines or pharmaceutical compositions

6 with suitable carriers such as physiological saline or other

7 injectable liquids. The vaccines or pharmaceutical compositions

8 comprising the modified antigens or the antigen-activated

9 dendritic cells of the invention would be administered in

10 therapeutically effective amounts sufficient to elicit an immune

11 response. Preferably, between about 1 to 100 micrograms of

12 modified antigen, or its equivalent when bound to dendritic

13 cells, should be administered per dose.

14   The present invention also provides a method and composition

15 for treating autoimmune disease. Such autoimmune diseases

16 include but are not limited to juvenile diabetes and multiple

17 sclerosis. Without being bound by theory, it is believed that

18 autoimmune diseases result from an immune response being directed

19 against "self-proteins", i.e., autoantigens that are present or

20 endogenous in an individual. In an autoimmune response, these

21 "self-proteins" are being presented to T cells which cause the T

22 cells to become "self-reactive". According to the method of the

23 invention, dendritic cells are pulsed with the endogenous antigen

24 to produce the relevant "self-peptide". The relevant self-

25 peptide is different for each individual because MHC products are

26 highly polymorphic and each individual MHC molecules might bind

27 different peptide fragments. The "self-peptide" may then be used

28 to design competing peptides or to induce tolerance to the self

29 protein in the individual in need of treatment.

30   Because dendritic cells can now be grown from precursors

- 24 -

1   according to the methods and principles identified here, and

2   because dendritic cells modify antigens to produce T cell

3   dependent immune responses the compositions of this invention are

4   particularly useful as infant vaccines. In addition, the

5   dendritic cells of the invention are suitable for producing

6   protein or peptide carriers which may be used to conjugate to

7   polysaccharide to produce effective conjugate vaccines for

8   infants which are T-cell dependent. A protein would be exposed

9   to the dendritic cells of the invention and allowed to be

10   modified by the dendritic cells. The modified protein would then

11   be purified and used to conjugate to polysaccharide to produce a

12   specific T-cell dependent vaccine suitable for infants.

13

14   **EXAMPLES**

15   Example 1.   Production of Mouse Dendritic Cells _In Vitro_
16   _From Proliferating Dendritic Cell Precursors_
17
18   **MATERIALS**

19   _Mice_: BALB/C, BALB/C x DBA/2 F1, BALB/C x C57BL/6 F1, C57BL/6 x

20   DBA/2 F1, and C57BL/6 males and females, 6-8 weeks of age were

21   purchased from Japan SLC Inc [Shizuoka, Japan], the Trudeau

22   Institute [Saranac Lake, NY], and Charles River Wiga [Sulzberg,

23   FRG]. Four preparations of rGM-CSF were evaluated with similar

24   results, the yield of dendritic cells reaching a plateau with 30-

25   100 U/ml. The preparations were from Dr. S. Gillis, Immunex

26   Corp, Seattle WA; Genetics Institute [supernatant from COS cells

27   transfected with mGM-CSF; used at 30U/ml or greater]; and Dr. T.

28   Sudo [supernatant from CHO cells transfected with the expression

29   vector, pHSmGM-CSF (22), and E.Coli expressed material].

30

31   _Blood_: Blood was obtained by cardiac puncture or from the

- 25 -

1 carotid artery. The blood was diluted in, or allowed to drip

2 into, RPMI-1640 with 100 U/ml heparin [about 2 ml/mouse]. Blood

3 cells were pelleted at 1000 rpm at 4°, resuspended in RPMI 1640,

4 and sedimented again. The pellet was suspended in 1 ml RPMI 1640

5 per mouse and mixed with an equal volume of 1.66% ammonium

6 chloride in distilled water to lyse the red cells. After 2 min

7 at room temperature, the suspension was spun at 1000 rpm at 4°.

8 The pellet, which still contained red cells, was resuspended

9 again in 0.5 ml RPMI and 0.5 ml NH₄Cl for 2 min, diluted in RPMI,

10 and sedimented again. After 2 more washes, most platelets and

11 red cells had been depleted and a population of blood leukocytes

12 had been obtained.

13

14 Aggregates of proliferating dendritic cells
15 from blood supplemented with GM-CSF
16
17   Blood leukocytes, usually from CxD2 F1 mice, were cultured

18 in 16 mm tissue culture wells [24 well dishes, Costar, #25820] in

19 medium (1 ml per well) supplemented with GM-CSF at 30U/ml and at

20 1.5x10⁶ cells/well. The medium was RPMI 1640 supplemented with

21 5% fetal calf serum [JRH Biosciences, Lenexa, KA], 50 uM 2-ME, 20

22 ug/ml gentamicin, and recombinant mouse GM-CSF. After overnight

23 culture, many monocytes adhered and the nonadherent cells were

24 transferred to new 16 mm wells. The adherent cells did not

25 develop dendritic cell colonies, but during the next week, the

26 nonadherent populations exhibited three changes. First, most of

27 the lymphocytes and granulocytes died or could be removed by

28 washing. Second, the surface of the well became covered with a

29 monolayer of tightly adherent cells that included macrophages and

30 fibroblasts. Third, affixed to scattered sites on the monolayer,

31 there developed small aggregates of cells. The cultures were fed

- 26 -

1   with GM-CSF (30 u/ml) at day 6-7 and then every 3 days by

2   aspirating 0.5-0.75 ml of the medium and adding back an equal

3   volume of fresh medium with GM-CSF. The aggregates continued to

4   expand in number and size. At about day 10, the cells were ready

5   to be subcultured. Any residual loose cells could be rinsed off

6   prior to dislodging the aggregates into fresh medium and GM-CSF.

7   About 0.8-1 million dislodged cells per original well were

8   divided into 3 subculture wells.

9   Most of the aggregates disassembled during this first

10   subculture, while the bulk of the adherent monolayer remained

11   attached to the original well. Upon transfer, most of the cells

12   in the dislodged aggregates adhered as single cells to the new

13   culture well but over a period of 2-3 days, aggregates

14   reappeared. The aggregates again were affixed to adherent

15   stromal cells, but these adherent cells were much less numerous

16   than the dense monolayer in the original culture. Over the next

17   4-7 days, aggregates filled the wells [Fig 2A]. These colonies

18   were often larger than those of the original wells and were

19   covered with many sheet-like processes typical of dendritic

20   cells. It was more difficult to count cells at this point, since

21   many of the aggregates contained a core of tightly associated

22   cells. However, the number of single cells that could be

23   recovered per well expanded about 2 fold between days 10 and 17

24   of culture.

25   If the cultures were allowed to overgrow, some cells with

26   the morphology of dendritic cells were released. More typically,

27   the cells were not allowed to overgrow and the aggregates were

28   dislodged and subcultured again at about 20 days. Prior to

29   subculture, the aggregates could be purified from free cells by

30   1g sedimentation. Such separations were more easily performed

- 27 -

1   with longer periods of culture, i.e., it was easier to isolate

2   intact aggregates at 3 vs. 2 vs. 1 week of culture. With

3   additional subculturing, the number of aggregates that were

4   produced per well was progressively reduced. However colonies of

5   growing cells, as confirmed by 3H-TdR labeling and

6   autoradiography [below], could be generated in subcultures for 1-

7   2 months. Following subculturing at 2-3 weeks, typical single

8   dendritic cells were now released into the medium [Fig 2B]. By

9   direct observation with video recording, these released cells had

10 the active motility of dendritic cells, continually extending and

11 retracting large veils or sheet-like processes. In the presence

12 of continued GM-CSF, one observed both free dendritic cells as

13 well as expanding colonies. In the absence of GM-CSF, only free

14 dendritic cells were released into the medium and colonies of

15 aggregates did not develop. The aggregates essentially fell

16 apart and did not reform. The yields of free dendritic cells per

17 subculture ranged from 0.3 - 2.5 x 10^5.

18   In summary, from a starting blood mononuclear culture of 1.5

19 x 10^6 cells, where dendritic cells were difficult to detect, we

20 on average obtained 5-10 subcultures each with at least 3-10x10^6

21 released dendritic cells at 3 weeks, as well as many aggregates

22 capable of further proliferation. Therefore aggregates of

23 growing cells were developing in mouse blood supplemented with

24 GM-CSF, and these aggregates were covered with dendritic cells

25 many of which could be released spontaneously into the medium.

26

27 Phenotype of the cell aggregates and dendritic cells
28 released therefrom
29
30   Cytospin preparations were made in a Shandon cytocentrifuge

31 using 3-10 x 10^6 cells. The slides were stored with desiccant

1   prior to fixation in acetone and staining with mAb followed by

2   peroxidase mouse anti-rat Ig [Boehringer Mannheim Biochemicals,

3   #605-545] or rabbit anti-hamster Ig [Accurate Chemical &

4   ·Scientific Corp, # JZY-036-003].   The preparations were stained

5   with Giemsa and mounted in Permount for bright field analysis.

6   For cytofluorography [FACScan, Becton Dickinson], aliquots of

7   cells were stained with primary rat or hamster mAb followed by

8   FITC mouse anti-rat Ig [Boehringer, #605-540] or biotin rabbit

9   anti-hamster Ig [Accurate, JZY-066-003] and FITC-avidin.

10   Cytospin preparations of 2-3 week cultures were examined

11   with a panel of mAb and an immunoperoxidase method. The released

12   cells, and many of the cells that could be dislodged from the

13   periphery of the aggregate, were similar in their stellate shape

14   and phenotype [Fig 3]. Most of the cells stained strongly with

15   mAb to MHC class II [Fig 3], the CD45 leukocyte common antigen,

16   CR3 receptor CD11b, and heat stable antigen [HSA, Fig 3], and

17   CD44. Staining with mAbs to the Fc receptor [2.4G2] and

18   macrophage F4/80 antigen [MAC, Fig 3] was weak or undetectable in

19   >95% of the cells. The cultures contained only rare B cells

20   [B220 mAb, RA-3], T cells [thy-1 mAb, B5-5], or granulocytes

21   [GRAN, mAb RB6; Fig 3]. Some cells at the periphery of the

22   aggregate, and many of the cells that were released from the

23   aggregates, were stained with two markers that are largely

24   restricted to dendritic cells. The interdigitating cell antigen

25   [mAb NLDC 145 (13), IDC; Fig 3], which also binds to thymic

26   epithelium, stained many but not all of the dendritic profiles.

27   Virtually all of the dendritic profiles stained with mAbs 2A1

28   [Fig 3] and M342 [Agger, R., Witmer-Pack M., Romani, N., Stossel,

29   H., Swiggard, W.J., Metlay, J.P., Storozynsky, E., Freimuth, P.,

30   and Steinman, R.M. Two populations of splenic dendritic cells

- 29 -

1 detected with M342, a new monoclonal to an intracellular antigen

2 of interdigitating dendritic cells and some B lymphocytes.

3 Submitted.] which stain granules in the perinuclear region of

4 mature dendritic cells as well as interdigitating cells in

5 sections through the T areas of lymphoid organs. Macrophages

6 from many sites [blood monocytes; peritoneal cavity macrophages;

7 macrophages in sections of lymph node, thymus, spleen] do not

8 contain 2A1 or M342-reactive granules.

9   Cytofluorography was used to gain semi-quantitative

10 information on the expression of antigens at the cell surface. A

11 panel of mAb were applied to two populations: cells that could be

12 dislodged from the aggregates by Pasteur pipetting, and cells

13 that were released spontaneously when the aggregates were

14 subcultured for 1 day. These "dislodged" and "released"

15 populations were identical in their dendritic shape and in

16 phenotype but for some exceptions that are considered below. The

17 phenotype of the released cells is shown in Fig 4, and the few

18 differences between aggregated and released cells are in Fig 5.

19 Virtually all the dendritic cells developing in and from the

20 aggregates expressed high levels of the leukocyte common [CD45,

21 mAb M1/9.3] and heat stable [mAbs M1/69 and J11d] antigens, as

22 well as high levels of CD44 [not shown] and CD11b [mAb M1/70].

23 Low levels of the following antigens were detected on the cell

24 surface: the dendritic cell antigen 33D1, the macrophage marker

25 F4/80, the Fcγ receptor antigen 2.4G2, the p55 IL-2 receptor CD25

26 antigen 3C7, and the CD11c integrin N418 [Fig 4]. These antigens

27 were noted on all cells by FACS even though many of the antigens

28 like F4/80 and 2.4G2 were weak or absent in the cytoplasm with an

29 immunoperoxidase method [Fig 3, above]. Several antigens were

30 absent: RB6 granulocyte, RA3 B cell, B5-5 thy-1, GK 1.5 CD4, and

1 SER-4 marginal zone macrophage [Fig 4].

2 Expression of class I and II MHC products by the dendritic

3 cells in these cultures was very high but nonetheless bimodal

4 [Figs 4 and Fig 5]. Most of the dendritic cells that were

5 dislodged from the aggregates had somewhat lower levels of MHC

6 class I and II, while dendritic cells that were released from the

7 aggregates had very high levels of MHC products. The other

8 marker that was different in the released and loosely attached

9 dendritic cells was NLDC 145 which was higher in the released

10 population [Fig 5, top panels]. We conclude that the phenotype

11 of the cells that arise from the proliferating aggregates is very

12 much like that seen in cultured dendritic cells from skin,

13 spleen, and thymus (24,28) with the exception that the M1/70

14 CD11b marker is more abundant.

15

16 3H-TdR autoradiography to verify growth of dendritic cell

17 precursors

18 After 2 and 3 weeks in liquid culture, the wells contained

19 numerous expanding aggregates of cells [as in Fig 2A], and in

20 some cases were already releasing nonadherent dendritic cells in

21 large numbers [Fig 2B]. Cultures were labeled with 3H-thymidine

22 to identify and phenotype the proliferating cells and their

23 progeny. For pulse labeling, 3H-TdR was added to the cultures [6

24 Ci/mM, 1 uCi/ml final]. 2h later, the medium was replaced with

25 3H-TdR free medium, and the cultures were separated into

26 nonadherent released cells and residual adherent aggregates for

27 examination on cytospin preparations [Shandon Inc, Pittsburgh PA,

28 #59900102]. The cytospin cells were stained for specific

29 antigens with mAb and immunoperoxidase as above. Also, the

30 slides were dipped in photographic emulsion [Kodak

- 31 -

1   autoradiography emulsion type NTB2 #165-4433) for exposure [5

2   days] prior to development, staining with Giemsa, and mounting in

3   Permount. For pulse chase experiments, a lower dose of 3H-TdR

4   was used to maintain cell viability, but the cells were handled

5   similarly otherwise. The pulse was applied at 0.1 uCi/ml for 2h

6   or for 16h, the latter to provide higher initial labeling

7   indices. The cells were washed and chased for 1-3 days prior to

8   harvesting and analysis as above with immunoperoxidase,

9   autoradiography, and Giemsa staining.

10   The 2 and 3 week cultures were exposed to 3H-TdR and

11 examined for proliferative activity. The labeled cells were

12 washed, spun onto slides, and the cytospins stained with mAb and

13 an immunoperoxidase method prior to dipping and exposure to

14 photographic emulsion. Important markers were mAbs 2A1 and NLDC-

15 145 which recognize intracellular granules and a cell surface

16 antigen in mature dendritic cells respectively [Fig 3, above].

17   When cultures were labeled with a 2h pulse of 3H-TdR, it was

18 apparent that the labeling index in the aggregates was very high,

19 at least 10-15% of the profiles in the aggregates being in S

20 phase [Fig 6, left and middle]. In contrast, if 3H-TdR was

21 applied to cultures that were releasing typical nonadherent

22 dendritic cells, the released fraction contained only rare

23 labeled profiles [not shown]. If GM-CSF was removed, 3H-TdR

24 labeling ceased within a day. Virtually all the 3H-TdR labeled

25 cells in the aggregate failed to label with mAb to markers found

26 on mature dendritic cells i.e., 2A1 and NLDC145 [Fig 6]. The

27 level of staining with anti-MHC class II mAb was less on the

28 cells in S-phase than in the released dendritic cell populations

29 [not shown].

30   Pulse chase experiments were then done to establish that

- 32 -

1   labeled cells in the aggregate were giving rise to typical

2   dendritic cells. Cultures were first exposed to a low dose of

3   3H-TdR, either for 2h or for 16h, the latter to label a larger

4   percentage of the cells in the aggregates. The wells were washed

5   free of radiolabel, and then the aggregates were dislodged and

6   separated from free cells by 1g sedimentation. The aggregates

7   were transferred to fresh medium without radiolabel, and over the

8   next 1-3 days of culture, many dendritic cells were released into

9   the medium. When the "chased" cultures were examined, several

10   findings were apparent. The labeling index remained high, i.e.,

11   most of the progeny of cells that were proliferating in the

12   aggregates were not being lost from the cultures. Second, the

13   grain counts were diluted several fold from those apparent in the

14   original pulse. Third, cells expressing the markers of mature

15   dendritic cells [NLDC-145, the 2A1 granular antigen, high levels

16   of MHC class II] were now radiolabeled [Fig 6 right]. Therefore

17   the cellular aggregates that GM-CSF was inducing in cultured

18   mouse blood were actively proliferating and releasing

19   nonproliferating progeny with many of the typical cytologic and

20   antigenic features of mature dendritic cells including the 2A1

21   granular antigen, the NLDC145 marker, and high levels of MHC

22   class II.

23

24   Accessory cell function for T cell proliferative responses

25   MLR stimulating activity was monitored in the GM-CSF treated

26   blood cultures. Cells from the blood cultures were exposed to

27   1500 rads [137Cs] and applied in graded doses to 3×10^5 purified

28   syngeneic or allogeneic T cells in 96 well, flat-bottomed

29   microtest wells. The T cells were nylon wool nonadherent, spleen

30   and lymph node suspensions that were treated with anti-Ia plus

- 33 -

1   Jlld mAbs and complement to remove residual APC. 3H-TdR uptake

2   was measured at 72-86 h [6 Ci/mM, 4 uCi/ml final].

3   Initially there was little or no MLR stimulating activity

4   [Fig 7, ★]. Some stimulating activity was noted at day 1 of

5   culture [Fig 7, O]. An examination of cytospin preparations

6   revealed that these 1 day nonadherent blood cells had a low

7   [<0.3%] but clear subset of Ia-rich, dendritic profiles. By day

8   7, when the proliferating aggregates were first evident on the

9   monolayer, the stimulating activity of the dislodged aggregates

10 had increased further, but was still 100 times less in specific

11 activity than typical dendritic cells [Fig 7, compare ▲ and ●]

12 even though most of the cells at day 7 and subsequent time points

13 were MHC class II positive. By day 14, at which time typical

14 nonadherent dendritic cells were just beginning to be released

15 from the aggregates, the nonadherent population had considerable

16 MLR stimulating activity, [Fig 7, ▼]. After 3 weeks, typical

17 mature dendritic cells had become abundant, and these indeed

18 stimulated comparably to their splenic counterparts [Fig 7,

19 compare ◊ and ●]. Other cells in the culture, such as those

20 dislodged from the aggregates, were about 10 fold less active

21 than dendritic cells [Fig 7, ◆]. We conclude that the aggregates

22 of prolifirating dendritic cells have some MLR stimulating

23 activity but that it is the mature released cells that are fully

24 potent, some 100-300 times more active on a per cell basis than

25 the populations in the starting culture at 1-7 days. During day

26 7-20 of culture, total cell numbers also expanded at least 5-10

27 fold.

28

29 Homing activity of dendritic cells in vivo

30   A second specialized feature of dendritic cells is their

- 34 -

1 capacity to home to the T areas of peripheral lymphoid tissues

2 (8,10). Dendritic cells or other cell types were labeled at 2-

3 $10x10^6$/ml with carboxyfluorescein for 10 min on ice [Molecular

4 Probes C-1157; 30 uM final concentration in Hanks balanced salt

5 solution (HBSS) with 5% FCS], washed in RPMI 1640, and injected

6 in a volume of 50 ul RPMI-1640 into the foot pads. Cne day

7 later, the draining popliteal lymph nodes were removed, frozen in

8 OCT medium, and sectioned [10 μ] in a cryostat. To sample the

9 entire node, we took duplicate specimens at regular intervals.

10 The sections were applied to multiwell slides [Carlson Scientific

11 microslides #111006], stored at -20°C, dried in a desiccator 30'

12 prior to use [or left at room temp overnight], fixed in acetone,

13 and stained with a peroxidase conjugated rabbit anti-FITC

14 antibody [Dakopatts, P404]. To verify that the dendritic cells

15 in the lymph node were in the T-dependent areas as described (8),

16 we added appropriate mAb to B cell, T cells, macrophages, or

17 dendritic cells and visualized the latter with alkaline

18 phosph.tase conjugated mouse anti-rat Ig [Boehringer Mannheim,

19 #605-5357] plus a chromogen kit [Biomeda Corp, Foster City CA

20 #S04]. We then blocked endogenous peroxidase with "Endo Blocker"

21 [Biomeda Corp, #M69] fcllowed by the peroxidase anti-FITC as

22 above.

23   Blood leukocytes, even when given at a dose of 10^6 cells per

24 footpad, failed to home to the lymphoid organ. When we tested

25 dendritic cells that had been generated with GM-CSF from blood,

26 homing to the T area was observed with injections cf 200,000

27 cells [Fig 8]. The selective localization to the T areas was

28 confirmed by double labeling the specimens with mAb that stain B

29 cells or T cells [Fig 8]. Therefore dendritic cells produced in

30 culture have the key functional features of this lineage: homing

- 35 -

1 to the T-dependent regions and strong accessory activity.

2

3 <u>Requirements for generating dendr. cell colonies from blood</u>

4   The surface phenotype of the bloo. 'l that gives rise to

5 the dendritic cell colonies was assessed by   ing the starting

6 population with antibodies and complement. Treat. :t with either

7 33D1 anti-dendritic cell, anti-MHC class II, or anti thy-1 did

8 not eliminate the colony forming unit [not shown]. Instead,

9 removal of thy-1* or Ia* cells enriched colony numbers several

10 fold. CSF's other than GM-CSF were also tested, either at the

11 start of the 1-3 week culture, or upon transfer of 2-3 week old

12 aggregates to form veiled cells [Fig 1]. None of the CSF's

13 tested, i.e., IL-3, M-CSF, G-CSF, SCF, supported the formation of

14 colonies or mature dendritic cells. Therefore the growing

15 dendritic colonies are very much dependent upon GM-CSF.

16   In an effort to identify proliferating precursors to the

17 dendritic cell system, we set up cultures from several t'ssues

18 that lacked mature dendritic cells and supplemented these with

19 different growth factors particularly the CSF's [M-CSF, G-CSF,

20 IL-3, GM-CSF, IL-1, and SCF]. Dendritic cell precursors were not

21 observed from neonatal epidermis, which contains mainly Ia'

22 Langerhans cells (29). To avoid overgrowth of granulocytes in

23 bulk bone marrow cultures which may make the identification of

24 typical cell colonies or large numbers of dendritic cells

25 difficult, it is preferred to remove the nonadherent,

26 proliferating granulocytes on days 2 and 4. Blood, which has few

27 typical dendritic cells in the mouse (30), proved to be very

28 effective for obtaining dendritic cell precursors. Growing cell

29 aggregates appeared after about 6 days in culture, and these were

30 often covered with profiles having the unusual and motile

1   processes of dendritic cells. With time, typical nonadherent

2   dendritic cells were released. The latter had the morphology and

3   movement of dendritic cells as previously described in cultured

4   mouse spleen, mouse skin, lymph from several species, and human

5   blood (25-27). Therefore to identify proliferating dendritic

6   cells, it seems critical to begin with an appropriate starting

7   population, preferably blood, and to supplement the culture with

8   GM-CSF.

9   Without wishing to be bound by any theory, we think that the

10 initial aggregates that appeared in the cultures represented

11 clones, since very small groups of 4-6 cells were observed early

12 on e.g., day 5. We tried to prove that the aggregates were

13 clonal by mixing blood cells from strains that were distinguished

14 with markers to polymorphic antigens like CD44 and MHC class II.

15 However we could not complete the experiments since we found that

16 mouse strains differed in the number and speed with which

17 colonies developed. BALB/C and DBA [and F1 strains derived

18 therefrom] were the most active; B6 and B10 were several times

19 less active; and strains like CBA/J, C3H/He, and A/J were poor

20 sources of proliferating, dendritic cell aggregates.

21   The precursors to the aggregates of proliferating dendritic

22 cells were not typical monocytes or dendritic cells, because the

23 number of aggregates that developed could be increased

24 substantially if one depleted monocytes by adherence or Ia-

25 positive cells with antibody and complement. Without wishing to

26 be bound by theory, we tentatively conclude that blood contains

27 an Ia-negative precursor that forms a proliferating aggregate.

28 In the aggregate, dendritic cells mature and are released as

29 nonproliferating progeny.

30   The formation of aggregates of dendritic cells required

1   exogenous GM-CSF. If the aggregates were placed in macrophage or

2   granulocyte-restricted CSF's [M-CSF, G-CSF], proliferation ceased

3   and neither macrophages nor granulocytes were formed. Because

4   the cultures contained macrophages and some stromal cells, in

5   addition to the dendritic cell aggregates, it was possible that

6   other cytokines were being produced that were critical to the

7   formation of dendritic cells. It appears however that the cells

8   in the aggregates have lost responsiveness to M- and G-CSF, and

9   that dendritic cells represent a distinct myeloid pathway of

10   development. Perhaps, without wishing to be bound by theory, the

11   pathway originates from a common precursor in which the dendritic

12   cell lineage is an offshoot that no longer responds to macrophage

13   and granulocyte restricted CSF's.

14   Labeling with 3H-thymidine, using pulse and pulse-chase

15   protocols, was important in establishing the precursor-product

16   relationships that were taking place in these liquid cultures

17   [Fig 6]. In a 2h pulse, virtually every labeled cell lacked two

18   typical markers of mature dendritic cells, i.e., the NLDC-145

19   interdigitating cell surface antigen (13) and the recently

20   identified 2A1/M342 granular cytoplasmic antigens [Agger, R.,

21   Witmer-Pack M., Romani, N., Stossel, H., Swiggard, W.J., Metlay,

22   J.P., Storozynsky, E., Freimuth, P., and Steinman, R.M. Two

23   populations of splenic dendritic cells detected with M342, a new

24   monoclonal to an intracellular antigen of interdigitating

25   dendritic cells and some B lymphocytes. Submitted.] These mAb

26   do not stain most macrophage populations that we have examined

27   either as isolated cells [blood, spleen, peritoneal macrophages]

28   or in sections [thymic cortex, spleen red pulp, lymph node

29   medulla]. In pulse chase protocols, large numbers of labeled

30   progeny were released from the aggregates, and these released

- 38 -

1 cells were nonadherent, motile, and strongly stimulatory in the

2 MLR. After combined autoradiography and immunoperoxidase

3 labeling, the labeled progeny carried the granular antigens, the

4 NLDC-145 antigen, and very high levels of MHC class II. Each of

5 these cytologic and antigenic markers are largely restricted to

6 dendritic cells.

7   Without wishing to be bound by theory, we believe that

8 maturation to typical nonproliferating dendritic cells occurred

9 within the aggregate. The aggregates were covered with cells

10 with the sheatlike or veiled processes of dendritic cells. Cells

11 with markers of mature dendritic cell markers [high MHC class II,

12 2A1 positive granules, NLDC antigen] were also observed at the

13 periphery of the cell aggregates. However, it was difficult to

14 isolate the aggregate intact, i.e., without dislodging these more

15 mature cells. The mechanism whereby dendritic cells matured and

16 left the aggregate was not clear. Maturation was enhanced in

17 older cultures [>2 weeks] or by removing adherent stroma cells.

18 Both proliferation and maturation was blocked if the cultures

19 contained too many fibroblasts.

20   The functional maturation that occurred in the proliferating

21 aggregate is striking. The dendritic cells that were generated

22 in culture were potent MLR stimulators. 100 dendritic cells

23 induced a much stronger primary MLR than 100,000 blood leukocytes

24 [Fig 7]. The increase in stimulating activity per Ia-positive

25 cell was at least 2 logs between the time that the aggregates

26 first appeared and the time that typical dendritic cells were

27 released in large numbers. Over this time period, cell recovery

28 increased 5-10 fold. Also the dendritic cell progeny homed in a

29 precise way to the T cell area of lymph node [Fig 8], another

30 functional property that was not detectable in blood cells [data

- 39 -

1 not shown].

2

3 Example 2.

4 Antigen activated dendritic cells as immunogens.

5   Dendritic cells prepared according to the method described

6 in Example 1 are plated at a concentration of approximately 1 x

7 10^5 cells per well of a 24 well plastic culture plate. The cells

8 are incubated in RPMI 1640 containing 5% fetal calf serum and GM-

9 CSF (30 u/ml). Antigen is added to the dendritic cell cultures

10 and the cultures are incubated with antigen for approximately 4

11 hours or for sufficient time to allow the dendritic cells to

12 handle the antigen in an immunologically relevant form, or in a

13 form that can be recognized by T cells. Such handling of the

14 antigen by the dendritic cells involves the dendritic cells 1)

15 acquiring, 2) processing, and 3) presenting the antigen to the T

16 cells in a form which is recognized by the T cells. Following

17 binding of the antigen to the dendritic cells the cells are

18 collected from the culture and used to immunize syngeneic mice.

19 The activated dendritic cells are injected subcutaneously into

20 the mice in an amount sufficient to induce an immune response to

21 the antigen.

22 Example 3.

23   Dendritic cells prepared as described in example 1 are

24 pulsed with a protein antigen for a time sufficient to allow the

25 dendritic cells to acquire, process and present the modified

26 antigen on the surface of the dendritic cells. The dendritic

27 cells are then collected from the culture for extraction of the

28 modified antigen.

29   For extraction of the modified antigen, the dendritic cells

30 are solubilized with detergent to extract the modified antigen

1   bound to MHC molecules. The MHC molecules bound to modified

2   antigen are purified by precipitation with antibodies which bind

3   the MHC molecules such as MH2. The modified antigens are

4   extracted from the precipitate for analysis.

5   While we have hereinbefore described a number of embodiments

6   of this invention, it is apparent that the basic constructions

7   can be altered to provide other embodiments which utilize the

8   methods and compositions of this invention. Therefore, it will

9   be appreciated that the scope of this invention is defined by the

10   claims appended hereto rather than by the specific embodiments

11   which have been presented hereinbefore by way of example.

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29

WHAT IS CLAIMED IS:

1.   A method of producing a population of dendritic cell
precursors from proliferating cell cultures comprising:

    a)   providing a tissue source comprising dendritic cell
precursors;

    b)   treating the tissue source to obtain a population of
cells suitable for culture _in vitro_;

    c)   culturing the tissue source on a substrate in a culture
medium comprising GM-CSF to obtain nonadherent cells and cell
clusters;

    d)   subculturing the nonadherent cells and cell clusters to
produce cell aggregates comprising proliferating dendritic cell
precursors;

    e)   serially subculturing the cell aggregates one or more
times to enrich the proportion of dendritic cell precursors.

2.   The method according to claim 1 wherein the tissue
source provided in step (a) is pretreated to reduce the
proportion of non-dendritic precursor cells.

3.   The method according to claim 2 wherein the tissue
source is blood or bone marrow and GM-CSF is present in the
medium at a concentration of about 1-1000 U/ml.

4.   The method according to claim 3 wherein the cell
aggregates of step (e) are serially subcultured one to five
times.

5.   The method according to claim 4 wherein the cell
aggregates are serially subcultured two to three times.

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6.   The method according to claim 5 wherein the cell aggregates are serially subcultured two times.

7.   The method according to claim 3 wherein the nonadherent cells and cell clusters of step (c) are subcultured after from about 0.3 to 1 day and the cell aggregates are serially subcultured every 3 to 30 days.

8.   The method according to claim 7 wherein the cell aggregates are serially subcultured every 10 to 20 days.

9.   The method according to claim 8 wherein the cell aggregates are serially subcultured every 20 days.

10.   The method according to claim 2 wherein the tissue source is blood or bone marrow, the nonadherent cells and cell clusters of step (c) are subcultured after about 0.3 to 1 day, the cell aggregates are serially subcultured one to five times every 3 to 30 days, and GM-CSF is present in the medium at a concentration of about 1-1000 U/ml.

11.   The method according to claim 10 wherein the nonadherent cells and cell clusters of step (c) are subcultured after about one half day and the cell aggregates are twice serially subcultured after 20 days.

12.   The method according to claim 3 wherein the culture medium is selected from the group consisting of RPMI 1640, DMEM, and α-MEM and wherein the culture medium is supplemented with serum.

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13. The method according to claim 12 wherein fetal calf serum is present in the culture medium in an amount of about 1 to 15%.

14. The method according to claim 13 wherein the fetal calf serum is present in the culture medium in an amount of about 10%.

15. The method according to claim 10 wherein the tissue source is blood and the concentration of GM-CSF in the medium is about 30-100 U/ml.

16. The method according to claim 10 wherein the tissue source is bone marrow and the concentration of GM-CSF in the medium is about 500-1000 U/ml.

17. A method of producing a population of mature dendritic cells from proliferating cell cultures comprising:

a) providing a tissue source comprising dendritic cell precursors;

b) treating the tissue source to obtain a population of cells suitable for culture in vitro;

c) culturing the tissue source on a substrate in a culture medium comprising GM-CSF to obtain nonadherent cells and cell clusters;

d) subculturing the nonadherent cells and cell clusters to produce cell aggregates comprising proliferating dendritic cell precursors;

e) serially subculturing the cell aggregates one or more times to enrich the proportion of dendritic cell precursors; and

f) continuing to culture the dendritic cell precursors for

a period of time sufficient to allow them to mature into mature dendritic cells.

18. The method according to claim 17 wherein the tissue source provided in step (a) is pretreated to reduce the proportion of non-dendritic precursor cells.

19. The method according to claim 17 wherein the tissue source is blood or bone marrow and GM-CSF is present in the medium at a concentration of about 1-1000 U/ml.

20. The method according to claim 19 wherein the cell aggregates of step (e) are serially subcultured one to five times.

21. The method according to claim 20 wherein the cell aggregates are serially subcultured two to three times.

22. The method according to claim 21 wherein the cell aggregates are serially subcultured two times.

23. The method according to claim 19 wherein the nonadherent cells and cell clusters of step (c) are subcultured after from about 0.3 to 1 day and the cell aggregates are serially subcultured every 3 to 30 days.

24. The method according to claim 23 wherein the cell aggregates are serially subcultured every 10 to 20 days.

25. The method according to claim 24 wherein the cell

aggregates are serially subcultured every 20 days.

26. The method according to claim 23 wherein the cell aggregates are serially subcultured one to five times.

27. The method according to claim 26 wherein the nonadherent cells and cell clusters of step (c) are subcultured after about one half day and the cell aggregates are twice serially subcultured after 20 days.

28. The method according to claim 19 wherein the culture medium is selected from the group consisting of RPMI 1640, DMEM, and α-MEM and wherein the culture medium is supplemented with serum.

29. The method according to claim 28 wherein fetal calf serum is present in the culture medium in an amount of about 1 to 15%.

30. The method according to claim 29 wherein the fetal calf serum is present in the culture medium in an amount of about 10%.

31. The method according to claim 19 wherein the tissue source is blood and wherein GM-CSF is present in the medium at a concentration of about 30-100 U/ml.

32. The method according to claim 19 where the tissue source is bone marrow and wherein the CM-CSF is present in the medium at a concentration of about 500-1000 U/ml.

33. A method for providing an antigen to a host comprising exposing an antigen to a culture of dendritic cells obtained according to the method of any one of claims 17, 19 or 31 to produce antigen-activated dendritic cells followed by inoculating the host with the antigen-activated dendritic cells.

34. The method according to claim 33 wherein the host is human.

35. A composition comprising dendritic cells prepared according to the method of any one of claims 17, 19 or 31.

36. A composition comprising antigen-activated dendritic cells wherein dendritic cells prepared according to claim 17 are pulsed with an antigen and wherein the dendritic cells process the antigen to produce a modified antigen which is expressed by the dendritic cells.

37. A composition comprising a dendritic cell modified antigen wherein a substance to be modified is exposed to a culture of dendritic cells prepared according to any one of claims 17, 19 or 31 and whereby the substance is modified by the dendritic cells to produce the modified antigen.

38. A method of immunizing against disease in humans or animals comprising, administering a vaccine comprising the composition of claim 36.

39. A vaccine comprising the composition of claim 36.

40. A method of immunizing against disease in humans or animals comprising, administering a vaccine comprising the composition of claim 37.

41. A vaccine comprising the composition of claim 37.

42. A method of treating autoimmune disease comprising administering to a person in need of treatment a therapeutically effective amount of the composition of claim 36 and wherein the antigen to be modified is a self-protein.

43. A method of treating autoimmune disease comprising administering to a person in need of treatment a therapeutically effective amount of the composition of claim 37 wherein the substance to be modified is a self-protein.

44. The method of claim 42 wherein the autoimmune disease is selected from the group consisting of multiple sclerosis and juvenile diabetes.

45. The method of claim 43 wherein the autoimmune disease is selected from the group consisting of multiple sclerosis and juvenile diabetes.

46. Dendritic cell precursors prepared according to the method of claim 1.

47. The dendritic cell precursors according to claim 46 wherein the tissue source is blood or bone marrow.

48. The dendritic cell precursor according to claim 47 wherein the tissue source is blood.

ABSTRACT OF THE DISCLOSURE

A method for producing proliferating cultures of dendritic cell precursors is provided. Also provided is a method for producing mature dendritic cells in culture from the proliferating dendritic cell precursors. The cultures of mature dendritic cells provide an effective means of producing novel T cell dependent antigens comprised of dendritic cell modified antigehs or dendritic cells pulsed with antigen which antigen is processed and expressed on the antigen-activated dendritic cell. The novel antigens of the invention may be used as immunogens for vaccines or for the treatment of disease. These antigens may also be used to treat autoimmune diseases such as juvenile diabetes and multiple sclerosis.